Investigating the role of endothelin receptor subtypes in the response to vascular injury

Nicholas Kirkby

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DECLARATION

I declare that this thesis and the work described within are solely my own work with the following exceptions: Mrs Susan Harvey performed picro-sirius red staining of injured femoral arteries. Dr Patrick Hadoke performed some wire- and ligation-injury procedures. I confirm that this thesis has not been previously submitted for any other degree.

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ABSTRACT

Neointimal hyperplasia, the proliferative growth of the innermost layer of the blood vessel wall, is a key process in the response to vascular injury, underlying conditions such as post-interventional restenosis and vein/arterial graft disease. One of the many mediators implicated in this process is endothelin-1 (ET-1), a potent vasoconstrictor with pro-inflammatory and pro-mitogenic actions, which acts through ETA and ETB receptor subtypes. It is well established that ET-1 increases, and ETA blockade reduces, neointima formation following vascular injury. The role of ETB is less clear because these receptors mediate potentially beneficial actions in endothelial cells (EC; such as nitric oxide production, and ET-1 clearance) but detrimental effects elsewhere (such as vascular smooth muscle) and it has been recently reported that non-cell-specific ETB deficiency is associated with increased neointimal lesion size following injury. The work described in this thesis addressed the hypothesis that endogenous ET-1 contributes to neointimal hyperplasia by activation of the ETA receptor, and that this action is moderated by concurrent activation of the ETB receptor expressed in EC.

The role of ET receptors in neointimal lesion development was assessed using two models of femoral arterial injury in the mouse: (i) an established method of intra-luminal wire-injury, and (ii) adaptation of a model of ligation injury that induces robust neointimal lesion formation without physical damage to the endothelium. Lesion development was assessed using standard histological techniques and this was augmented by development of quantitative optical projection tomography (OPT) to allow three-dimensional analysis of lesions.

The role of ETA and ETB receptors in these models was addressed using suitable pharmacological ET receptor antagonists. Following wire-injury, selective ETB blockade (A192621; $30mg.kg^{-1}.day^{-1}$; 35 days) increased lesion size and blood pressure without significant altering lesion composition. In contrast, selective ETA blockade (atrasentan; $10mg.kg^{-1}.day^{-1}$; 35 days) reduced lesion size and blood pressure. Combined ETA+ETB antagonism had no effect on lesion size, despite

reducing blood pressure, and reducing collagen content of the lesions. In the ligation model, neither ETA selective, ETB selective nor ETA+ETB blockade altered lesion size as assessed by standard histology but analysis by OPT indicated that ETA blockade, with or without concurrent ETB blockade, reduced lesion volume.

The influence of ETB receptors expressed by ECs on lesion formation was addressed using EC-specific ETB knockout mice. Small vessel myography indicated that endothelium-dependent relaxation was unaltered in femoral arteries from these mice. In addition, no effect on lesion size or rate of development was observed in either wire- or ligation-injury models of neointima formation (although subtle effects on lesion and medial composition were apparent after intra-luminal injury).

These results indicate that ETB receptor activation can moderate the detrimental actions of the ETA receptor on neointimal lesion progression, and that this role is dependent on the mode of vascular injury. Furthermore, in this setting, this beneficial action is not primarily mediated by ETB expressed by EC, suggesting that ETB in other cell types can reduce lesion development through another, unidentified mechanism. Therefore, while both ETA selective and non-selective ETA/B antagonists are currently in clinical use, in conditions where similar arterial remodelling processes occur, selective ETA receptor antagonists might be preferred.

PRESENTATIONS AND PRIZES

Oral presentations

Endothelial cell-specific ablation of the endothelin-B receptor does not alter expression or functional response of the endothelin-A receptor

Scottish society for experimental medicine, Edinburgh, November 2006.

ETB-dependent inhibition of neointimal proliferation is not mediated by receptors expressed on the endothelium

11th international conference on endothelin, Montreal, September 2009.

Poster presentations

Endothelial cell specific endothelin-B receptor knockout does not impair endothelial function in mouse femoral arteries

Centre for cardiovascular science symposium, Edinburgh, July 2006.

Endothelial cell-specific endothelin-B receptor knockout does not impair endothelium-dependent vasodilatation in the mouse femoral artery

10th international conference on endothelin, Bergamo, September 2007.

The role of endothelin receptor subtypes in the response to vascular injury: histological and optical projection tomographic examination

Scottish cardiovascular forum, Inverness, January 2009.

The role of endothelin receptor subtypes in the response to vascular injury: histological and optical projection tomographic examination

Centre for cardiovascular science symposium, Edinburgh, June 2009.

Prizes

Student poster prize

Centre for cardiovascular science symposium, Edinburgh, June 2009.

American Physiological Society research recognition award

11th international conference on endothelin, Montreal, September 2009.

PUBLICATIONS

Review publications

Kirkby, N.S., Hadoke, P.W., Bagnall, A.J., and Webb, D.J. (2008). **The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house?** British Journal of Pharmacology *153*, 1105-1119.

Original research publications

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACh	Acetylcholine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
AQP2	Aquaporin 2
αSMA	Alpha-smooth muscle actin
BABB	Benzyl alcohol:benzyl benzoate
bFGF	Basic fibroblast growth factor (fibroblast growth factor-2)
BH_4	Tetrahydrobiopterin
BP	Blood pressure
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CCD	Charge coupled device
CCL2	Chemokine ligand 2 (monocyte chemoattractant protein-1)
CCR2	Chemokine receptor 2
CD31	Platelet-endothelial cell adhesion molecule-1
CRC	Concentration response curve
Cre	Cyclisation recombination
CRP	C-reactive protein
CT	Computed tomography
DAB	3,3'-diaminobenzidine
dATP	2’-deoxyadenosine 5’-triphosphate
dCTP	2’-deoxycytosine 5’-triphosphate
dGTP	2’-deoxyguanine 5’-triphosphate
DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
DPX	Di-n-butyl phthalate in xylene.

dTTP	2’-deoxythymidine 5’-triphosphate
EC	Endothelial cell
ECE	Endothelin converting enzyme
EDHF	Endothelium-derived hyperpolarising factor
EDTA	Ethylene diamine tetraacetic acid
EEL	External elastic lamina
EGF	Epidermal growth factor
E_{max} (%)	Maximum response as % response to KPSS
E_{max} (mN/mm)	Maximum response as absolute value
eNOS	Endothelial nitric oxide synthase (NOS3)
eOPT	Emission optical projection tomography
ET	Endothelin
ETA	Endothelin-A receptor
ETB	Endothelin-B receptor
$F(ab)_2$	Fragment - antigen binding
GFP	Green fluorescent protein
GTP	Guanine triphosphate
Hph-1	Hyperphenylalaninemia-1
ICAM-1	Intercellular adhesion molecule-1
IEL	Internal elastic lamina
iNOS	Inducible nitric oxide synthase (NOS2)
KPSS	125mM KCl, iso-osmolar physiological salt solution
LDL	Low density lipoprotein
LMP	Low melting point
L-NAME	N-nitro-L-arginine-methyl-ester
loxP	Locus of X-over P1
LP	Low pass
LSAB	Labelled streptavidin biotin
Mac-1	Macrophage-1 antigen (complement receptor-3)
Mac-2	Macrophage-2 antigen (galectin-3)
MCP-1	Monocyte chemoattractant protein-1 (chemokine ligand 2)
M-CSF	Macrophage-colony stimulating factor

MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-B
nNOS	Neuronal nitric oxide synthase (NOS1)
NO	Nitric oxide
NOS	Nitric oxide synthase
OPT	Optical projection tomography
PAH	Pulmonary arterial hypertension
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
pD_2	$-\log(EC_{50})$
PDGF	Platelet-derived growth factor
PE	Phenylephrine
PECAM-1	Platelet-endothelial cell adhesion molecule-1 (CD31)
PET	Positron emission tomography
PGI_2	Prostaglandin I_2 (prostacyclin)
PIER	Protease-induced epitope retrieval
PKC	Protein kinase C
PLC	Phospholipase C
P-selectin	Platelet-selectin
PSS	Physiological salt solution
PTCA	Percutaneous coronary transluminal angioplasty
RAAS	Renin angiotensin aldosterone system
S6c	Sarafotoxin S6c
SBP	Systolic blood pressure
SEM	Standard error of the mean
SNP	Sodium nitroprusside
SPECT	Single photon emission computed tomography

TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
TGFβ	Transforming growth factor-beta
TNFα	Tumour necrosis factor-alpha
tOPT	Transmission optical projection tomography
TXA_2	Thromboxane A_2
UST	United States trichrome
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

Chapter 1

Introduction

The impact of cardiovascular disease is difficult to over-state – it is the leading cause of death in Europe, North America and parts of Asia (Ross, 1999). Some remodelling of vascular structure is a common feature of these conditions and, whilst this may be beneficial in some cases, exuberant, dysfunctional or otherwise inappropriate remodelling is strongly implicated in disease progression. In particular, acute or chronic injury to the vascular wall initiates inflammatory and proliferative processes, which lead to the development of the intimal lesions that define atherosclerosis, post-interventional restenosis and vascular graft disease. The discovery of endothelin-1 (ET-1; Yanagisawa *et al.*, 1988) was rapidly followed by the realisation that its release could contribute to the pathogenesis of a variety of cardiovascular diseases (reviewed by Haynes & Webb, 1992; Rubanyi & Polokoff, 1994). Considerable research effort has since revealed ET-1 to be a major mediator of vascular remodelling processes (section 1.3). Indeed, in addition to its potent vasomotor activity, ET-1 promotes many responses critical to intimal lesion development, including: inflammation, mitogenesis and extracellular matrix turnover. Further, an extensive literature describes the ability of blockade of the ET system to moderate lesion development in models of acute vascular injury. What is less clear, however, is the individual role of ET receptor subtypes (ETA and ETB) in this process. Whilst ETA mediates almost entirely detrimental actions in disease, ETB receptor stimulation may evoke actions that both diminish and enhance disease progression. This is of considerable importance because both ETA-selective and non-selective ETA/B antagonists are under investigation for a variety of clinical indications yet the relative merits of these treatment strategies is still the subject of debate. The work described in this thesis therefore addresses the role of ET receptor subtypes in regulation of neointimal lesion formation. Consequently, this introduction will consider the pathogenesis of neointimal proliferation, the biology of the ET system, and how their interaction may contribute to vascular remodelling.

1.1 VASCULAR INJURY AND REMODELLING

The vascular system comprises an integrated network of arterial and venous conduits, the primary function of which is the distribution of nutrients, heat, waste and endocrine messengers through the body. Whilst the heart ultimately drives this

process, the vascular wall crucially smoothes cardiac output by arterial compliance, regulates blood flow, blood pressure and organ perfusion by adjusting luminal diameter, and through changes in permeability controls nutrient flux.

1.1.1 *Structure of the vascular wall*

The structure of the vascular wall varies widely between arterial, capillary and venous vessels and can comprise up to three distinct layers – the *tunica intima*, *tunica media* and *tunica adventitia*.

1.1.1.1 *Tunica intima*

This innermost layer of the vascular wall is present in all vessels from large arteries to micro-vascular capillaries. It typically comprises a confluent monolayer of endothelial cells (EC) anchored to a basement membrane, although may contain interspersed vascular smooth muscle cells (VSMC) and additional layers of extracellular matrix. It is the only component of the capillary wall, and in larger vessels is delineated by the internal elastic lamina. The capillary endothelium allows passage of substances between the vascular and tissue compartments, under the regulation of various signals, including: cytokines (Bucana *et al.*, 1988; Horvath *et al.*, 1988), histamine and bradykinin (Majno *et al.*, 1969). The endothelium also provides an anti-thrombotic coating to the vessel wall, reflecting expression of heparin-like anti-coagulant proteoglycans (Marcum *et al.*, 1986) and enzymes that degrade pro-aggregants (Cooper *et al.*, 1979) or activate plasminogen (Kester, 1969). The groundbreaking description of an endothelium-derived relaxing factor by Furchgott and Zawadzki (1980) demonstrated that endothelial cells play a pivotal role in control of vascular tone. It is now known that the endothelium generates a variety of vasodilator factors: nitric oxide (NO; Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987), prostacyclin (PGI2; Weksler *et al.*, 1977), endothelium-derived hyperpolarising 'factor' (Chen *et al.*, 1988; Nagao & Vanhoutte, 1992; Busse *et al.*, 2002) and vasoconstrictor factors: thromboxane A_2 (TXA_2; Moncada *et al.*, 1976; Ingerman-Wojenski *et al.*, 1981) and ET-1 (Yanagisawa *et al.*, 1988). Endothelium-derived vasoactive factors have several additional actions. NO, suppresses VSMC

mitogenesis (Garg & Hassid, 1989), reduces platelet aggregation (Radomski *et al.*, 1987b, 1987a), and inhibits leucocyte chemotaxis and adhesion (Bath *et al.*, 1991; Hickey & Kubes, 1997). Similarly, PGI_2 also inhibits both proliferation (Sinzinger *et al.*, 1987) and platelet aggregation (Moncada *et al.*, 1977; Whittle *et al.*, 1978). In contrast, vasoconstrictors such as ET-1 may actively stimulate cell proliferation and inflammation (see 1.3.1). The synthesis and release of these EC-derived vasoactive substances is controlled by physicochemical stimuli (shear stress, wall stretch) and a variety of humoural substances (bradykinin, cytokines, catecholamines), and this allows acute regulation of luminal calibre in muscular vessels.

1.1.1.2 *Tunica media*

The *tunica media* contains circumferentially-organized layers of VSMCs interspersed with collagen and elastin fibrils and elastic laminae, the outermost of which (internal and external elastic laminae) define its borders. A principal function of the medial layer is the control of vessel diameter which is achieved by regulation of smooth muscle tone. The *tunica media* is absent in capillaries, and negligible in all but the largest veins. In arteries, however, it is substantial and its composition varies depending on the position of the artery within the vascular tree. In large elastic vessels such as the aorta, the media is rich in elastic fibres and relatively poor in smooth muscle, resulting in compliant vessels capable of accommodating and smoothing pulsed cardiac output. 'Resistance' vessels by comparison have a VSMC-rich media affording greater regulation of blood flow and pressure (Berne & Levy, 2000).

1.1.1.3 *Tunica adventitia*

Beyond the external elastic lamina, both in arteries and veins, lies the *tunica adventitia*. This is primarily composed of extracellular matrix components, especially collagen, but may also contain intermittent VSMCs, fibroblasts, resident macrophages and the sympathetic-innervation to the vascular wall. It provides mechanical strength and stability to the vessel and anchors it in the surrounding tissue. In large vessels, the adventitial layer contains microvasculature (*vasa*

vasorum) and lymphatic vessels and, therefore, serves to prevent hypoxia where the metabolic needs of the vascular wall are not served by blood in the vessel lumen (Berne & Levy, 2000).

1.1.2 *Vascular remodelling*

Rather than being determined solely at the time of development, vascular structure can dynamically respond to prevailing conditions. Chronically-elevated laminar shear stress (e.g. induced by an arterio-venous fistula or luminal stenosis) elicits dilation, wall stretch associated with hypertension promotes inward growth of the vessel wall (either by contraction or by medial thickening) and hypoxia initiates angiogenic processes leading to the formation of new capillary beds. The term vascular remodelling, therefore, refers to a variety of structural changes that occur as a result of alterations to the growth, death and migration of vascular cells and the balance between extracellular matrix synthesis and degradation (Gibbons & Dzau, 1994). Some manner of vascular remodelling is associated with most cardiovascular diseases. This can be beneficial (as in the case of outward dilation in atherosclerosis) or detrimental (for example, medial hypertrophy in pulmonary hypertension). The 'response to injury' represents a special case of vascular remodelling in which lesions develop in the intimal layer of the vessel wall (**Figure 1-1**). This response can be further sub-divided according to the nature of injury – chronic (coronary heart disease, peripheral vascular disease, cerebrovascular disease) or acute (post-interventional restenosis, vascular graft disease). Whilst the term 'neointimal lesion formation' and derivatives technically describe responses to both chronic and acute vascular injury, for clarity it is reserved solely for the latter in this thesis.

1.1.2.1 *Chronic injury*

Chronic injury to the vascular wall, over decades, results in formation of atherosclerotic plaques – complex intimal lesions of heterogeneous composition which may include macrophage-foam cells, VSMCs, free cholesterol esters, thrombus and calcium deposits. The development of atherosclerosis is typically asymptomatic but the disease can dramatically manifest, particularly when plaques

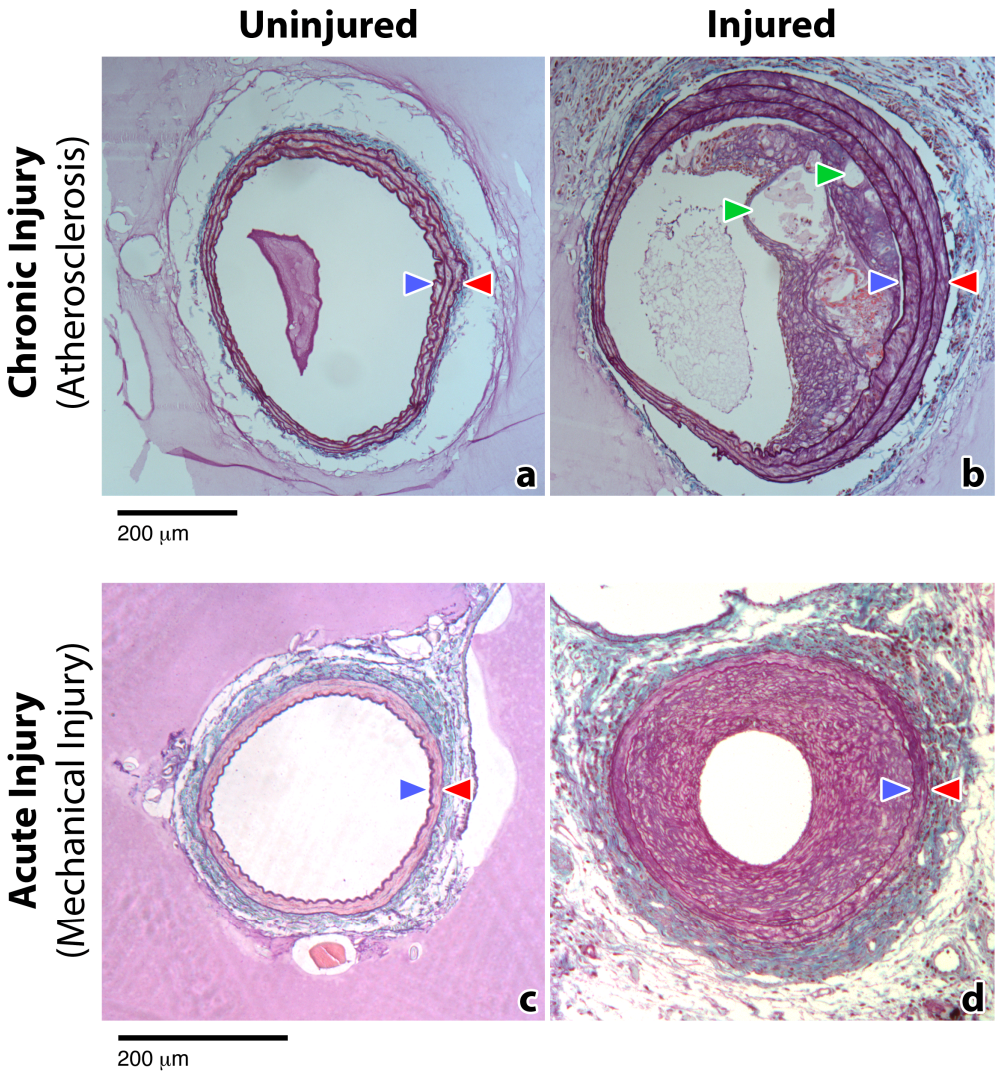


Figure 1-1 Structure of the arterial wall.

Histological sections of healthy mouse brachiocephalic (a) and femoral arteries (c), the brachiocephalic arteries of apoE$^{-/-}$ deficient atherosclerotic mice (b), and the mouse femoral artery following intra-lumenal wire injury (d) stained by the United States Trichrome method. The arterial wall is composed of three layers. The intima lies within the internal elastic lamina (blue arrowheads), the media likes between the internal (blue arrowheads) and external elastic laminae (red arrowheads) and the adventitia lies beyond the external elastic lamina (red arrowheads). Chronic (atherosclerosis) and acute (mechanical) vascular injury elicits formation of lesions in the intimal layer of the vessel wall. The lesions induced by both injuries contain smooth muscle cells and extracellular matrix, but only in the atherosclerosis are intra-lesion lipid pools apparent (green arrowheads).

are eroded or otherwise rupture, revealing their thrombogenic contents. The resulting intravascular thrombosis (or its embolisation) may cause infarction, most catastrophically in the coronary or cerebral circulations, and this is the cause of substantial morbidity and mortality. Indeed, coronary heart disease and stroke accounted for 27% of all deaths in the UK in 2006 (www.heartstats.org). A perhaps more benign consequence of atherosclerosis is development of luminal stenoses (or vasospasm) that limit blood flow through the affected vessel sufficiently that the metabolic needs of the tissue distal to it are not met. This results in tissue hypoxia and pain, typically on exertion, and again, commonly affects the coronary vasculature (angina pectoris). Since the work described in this thesis is concerned with the processes involved in the response to acute injury, the pathogenesis of atherosclerosis will not be described in detail. Further information can be found in many excellent reviews (Ross, 1999; Lusis, 2000; Weissberg, 2000).

1.1.2.2 *Acute injury*

Although life-style modification and pharmacotherapy may retard atherogenesis, mechanical re-vascularisation procedures have proven invaluable for the treatment of advanced disease. Interventions such as percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG) restore patency to stenosed or thrombosed arteries but, in doing so, inevitably cause a degree of damage to the target vessel. This acute injury results in a rapid and resolving process of intimal lesion formation (~1 month in animal models, 3-7 months in man). The 'neointimal lesions' resulting from acute injury differ substantially from those in atherosclerosis - they are rich in VSMC and extracellular matrix and are inherently stable. Neointima formation can be considered an appropriate response to acute injury, in which the vessel heals, gains mechanical strength and recovers its endothelial coating. An over-exuberant response to this acute injury, however, results in a neointimal lesion that can itself compromise vessel patency and this represents a major limitation in the ultimate success of these procedures.

1.1.2.2.1 Percutaneous coronary interventions

First achieved in man by Andreas Gruntzig (Gruntzig *et al.*, 1978), PTCA and its derivatives have become the most common interventions for the treatment of coronary artery stenosis, with over 70,000 performed in the UK in 2006 (BHF; www.heartstats.org). The procedure entails the dilation of the stenosed artery by intra-luminal inflation of a balloon, introduced via guide wire through the arterial circulation from a femoral arteriotomy. Whilst balloon inflation is traumatic to the vascular wall, this action serves to increase luminal cross-section by several possible means, including: outward distension of the arterial wall, axial re-distribution of plaque contents and plaque embolisation (Wolf *et al.*, 1984). Simple PTCA is associated with a relatively high incidence of failure (~30%) which primarily reflects subsequent re-occlusion of the dilated vessel and the need for further re-vascularisation interventions. Re-occlusion of dilated vessels ('restenosis') occurs via several mechanisms, including: acute elastic recoil (Rensing *et al.*, 1990), structural contraction of the vessel wall and formation of neointimal lesions (Schneider & Gruntzig, 1985). To address these problems, several refinements to the technique have been developed. Most important among these has been the introduction of vascular stents; support structures expanded by the angioplasty balloon which remain in the stenosed artery (Sigwart *et al.*, 1987) and substantially reduce the need for re-vascularisation (from 24.5% to 15% at 7 months in the Benestent trial; Serruys *et al.*, 1994). While stent implantation reduces elastic recoil and structural contraction (Haude *et al.*, 1993), the problem of neointima formation ('in-stent restenosis') is intensified due to the severity of injury and persistence of a foreign body within the arterial wall (Schwartz *et al.*, 1992b). A more recent concept is of the drug-eluting stent, wherein the implanted device is coated with a pharmacological agent to inhibit neointima formation, which is slowly released into the vessel wall. Pivotal trials of the CYPHER® sirolimus-eluting stent indicate a reduction in the need for re-vascularisation of the stented lesion from 16.6% to 4.1% at 270 days (Moses *et al.*, 2003). Although drug-eluting stents have been enthusiastically adopted, concerns have been raised regarding late (>1 year) thrombotic complications, probably reflecting powerful inhibition of this healing response. This necessitates extended anti-platelet therapy and possibly results in

increases in mortality, especially when used for off-label indications (Liistro & Colombo, 2001; McFadden *et al.*, 2004; Camenzind *et al.*, 2007).

1.1.3 *Mechanisms of neointima formation*

The formation of a neointimal lesion is the result of elaborate interactions between cells of the vascular wall, leucocytes and circulating progenitor cells. The commonly accepted 'cascade model' by which this occurs was proposed by Libby *et al* (1992). In this model, following vascular injury and EC damage/denudation, platelets adhere to the sub-endothelial surface and release a cocktail of mediators: notably including many VSMC mitogens. Medial and adventitial trauma and presentation of adhesion molecules by platelets also results in recruitment of leucocytes to the region of injury, which in turn, release further chemoattractants and mitogens initiating a self-amplifying inflammatory response. Under mitogenic and chemotactic pressure from these events at the luminal surface, medial VSMCs proliferate and migrate towards the intima, where they adopt a synthetic phenotype. These intimal VSMCs also replicate *in situ* and deposit extracellular matrix, under the control of mitogens released from leucocytes and VSMCs themselves. Eventually, as replicating cells become quiescent, and the inhibitory properties of the luminal endothelium recover, lesion growth is slowed and a mature, VSMC-rich neointima remains. The individual processes contributing to neointima formation are further considered below:

1.1.3.1 *Role of thrombosis*

Thrombosis is an obvious candidate for an initiating event in neointima formation. The endothelium, which actively prevents thrombosis by release of inhibitors of platelet aggregation and catabolism of pro-aggregants, is damaged or destroyed by acute mechanical injury, and the thrombogenic sub-endothelial layer is revealed. Platelets secrete a variety of mitogens/chemoattractants such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ) and thrombin and present leucocyte adhesion molecules. Further, it has been suggested that mural thrombus formation provides a framework into which VSMCs and leucocytes can migrate and proliferate to form a neointima (Schwartz *et al.*, 1992a). Whether

platelet activation and thrombosis are obligatory steps in neointima formation is open to question. Certainly, anti-thrombotic treatments reduce lesion formation in some models of neointima formation (Clowes & Karnowsky, 1977; Clopath, 1980; Buchwald *et al.*, 1996). The role of thrombosis, however, varies between species and models, for example, being important in pigs but minimal in most rodent models (Schwartz *et al.*, 2004). As such, the most damning evidence comes from the clinic. Numerous treatments that interfere with platelet activation/coagulation appear unable to inhibit restenosis in man including ADP receptor antagonists (Kastrati *et al.*, 1997), direct thrombin inhibitors (Bittl *et al.*, 1995; Serruys *et al.*, 1995), heparins (Karrillon *et al.*, 1996), heparin-eluting stents (Serruys *et al.*, 1996), aspirin/dipyridamole (Schwartz *et al.*, 1988), epoprostanol (Gershlick *et al.*, 1994) and possibly glycoprotein IIb/IIIa antagonists (Lefkovits *et al.*, 1996; Gibson *et al.*, 1998; Kastrati *et al.*, 2004). Indeed, the only anti-platelet agent that has been consistently shown to reduce the incidence of post-interventional restenosis is cilostazol (Douglas *et al.*, 2005), and this response is easily attributable to the anti-proliferative action of the molecule (Takahashi *et al.*, 1992). Thrombosis, therefore, does not appear to be critical to neointima formation, at least in man. Regardless, post-interventional anti-platelet therapy has been widely adopted for the prevention of acute peri- and post-interventional thromboembolic events (Schwartz *et al.*, 1988).

1.1.3.2 *Role of inflammation*

A role for inflammatory processes in neointima formation is more certain. Animal models suggest that neutrophil accumulation occurs within 24 hours of acute vascular injury with macrophage infiltration apparent within 14 days (Cole *et al.*, 1987; Tanaka *et al.*, 1993). These cells release a variety of molecules that could contribute to the formation of a neointima including PDGF (Shimokado *et al.*, 1985), tumor necrosis factor-α (TNFα; Matthews, 1981), interleukin-1 (IL-1), interleukin-6 (IL-6), ET-1 and matrix metalloproteinase (MMPs). The release of these growth factors, cytokines and matrix remodelling enzymes enables the proliferation and migration of medial VSMCs and amplifies the inflammatory signal (Libby *et al.*, 1992). The importance of inflammatory processes in neointima formation is demonstrated by the dramatic reductions in lesion size associated with depletion of

leucocytes by any of several methods (Miller *et al.*, 2001; Wolff *et al.*, 2004). Indeed, in a rat carotid artery balloon-injury model, liposomal bisphosphonate administration reduced lesion size by >70% (Danenberg *et al.*, 2003). Moreover, numerous specific disruptions of inflammatory pathways also result in reduced lesion size following acute vascular injury. These include the adhesion molecules P-selectin (Kumar *et al.*, 1997; Hayashi *et al.*, 2000), vascular cell adhesion molecule-1 (VCAM-1; Oguchi *et al.*, 2000) and intercellular adhesion molecule-1 (ICAM-1; Zou *et al.*, 2000), subunits of the pro-inflammatory transcription factor nuclear factor-κB (NFκB; Squadrito *et al.*, 2003; Ruusalepp *et al.*, 2006), macrophage chemoattractant protein-1 (MCP-1; Furukawa *et al.*, 1999; Schepers *et al.*, 2006), and its receptor CCR2 (Roque *et al.*, 2002). Further, deletion or blockade of the IL-1 receptor (Chamberlain *et al.*, 2006), TNFα (Zimmerman *et al.*, 2002) or infusion of the anti-inflammatory cytokine, interleukin-10 (IL-10; Feldman *et al.*, 2000) also reduces neointima formation.

Data from clinical studies suggest this insight from animal models is applicable to man. Macrophages are abundant in atherectomy (Kornowski *et al.*, 1998) and post-mortem specimens (Komatsu *et al.*, 1998) of post-interventional re-stenotic lesions. Plasma concentrations of inflammatory markers including MCP-1, IL-6, IL-1β, macrophage-colony stimulating factor (M-CSF) and C-reactive protein (CRP) are also increased following PCI, and those of M-CSF and CRP predict the incidence of restenosis (Pietersma *et al.*, 1995; Buffon *et al.*, 1999; Hojo *et al.*, 2001; Kochiadakis *et al.*, 2007). Similarly, there is evidence of rapid neutrophil activation following PTCA including increased circulating levels of elastase (De Servi *et al.*, 1990) and lactoferrin (Steg *et al.*, 1993). Isolated neutrophils from these patients also show increased expression of Mac-1, the level of which predicts the incidence of subsequent restenosis (Neumann *et al.*, 1996; Serrano *et al.*, 1997; Inoue *et al.*, 2003). Stents that elute sirolimus and its analogues are known to inhibit post-interventional restenosis (Moses *et al.*, 2003) and this may be, in part, a reflection of their immunosuppressive properties (Calne *et al.*, 1989). Indeed, sirolimus eluting stents reduce the acute post-procedural increase in inflammatory markers that follows bare metal stent implantation (Kochiadakis *et al.*, 2007).

1.1.3.3 *Role of VSMCs and matrix deposition*

The role of VSMCs in neointima formation is also difficult to doubt. These cells are the major constituent of mature neointimal lesions in animal models (Clowes *et al.*, 1983; Zwolak *et al.*, 1987; Groves *et al.*, 1995) and post-interventional restenotic lesions (Essed *et al.*, 1983; Ueda *et al.*, 1991; Komatsu *et al.*, 1998). In addition to providing neointimal mass, VSMCs actively contribute to lesion development by the expression of adhesion molecules, and secretion of cytokines and mitogens that amplify the inflammatory and proliferative response (Libby *et al.*, 1992; Lindner & Reidy, 1993; Lindner, 1995; Tanaka *et al.*, 1996; Crook *et al.*, 2000). They are defined as 'smooth muscle' by their spindle-like morphology and immunoreactivity for α-smooth muscle actin but, in the neointima they commonly also express abnormal myosin chain subtypes (Leclerc *et al.*, 1992) and demonstrate reduced expression of contractile proteins (Ueda *et al.*, 1991). This switch from a 'contractile' to a 'synthetic' phenotype is similar to that seen in cultured VSMCs (Chamley-Campbell *et al.*, 1979) and is probably a response to various stimuli, including: stretch of the vessel wall (Birukov *et al.*, 1995), endothelial denudation (Chamley-Campbell & Campbell, 1981), exposure to platelet and leucocyte products, and remodelling of the medial extracellular matrix (Thyberg, 1998). This synthetic phenotype is also characterised by hypersensitivity to mitogens and increased secretion of extracellular matrix (Chamley-Campbell *et al.*, 1979; Chamley-Campbell *et al.*, 1981), and thus, is considered to be important in the pathogenesis of neointima formation.

The kinetics of cell proliferation in neointimal lesion growth has been the subject of detailed study in animals. In the media, proliferation, presumably primarily of VSMC-like cells, peaks 2-3 days after injury, but in models featuring more severe medial damage, this may be as late as 14 days. In the intima, proliferation peaks between 7 and 14 days, and may remain high for long periods. The VSMC content of lesions appears to reach a stable maximum between 21 and 28 days and this is consistent between models of PCI and vein graft hyperplasia (Clowes *et al.*, 1983; Zwolak *et al.*, 1987; London & Mayberg, 1994; Groves *et al.*, 1995; Reis *et al.*, 2000). These values support the idea that medial VSMC proliferation precedes

migration to, and proliferation in, the intima (Spaet *et al.*, 1975; Lee *et al.*, 1993). Both proliferation and migration of VSMCs are likely to be driven by mitogens released from leucocytes and from platelets adhering to the injured luminal surface. It has also recently been suggested that neointimal VSMCs can be derived from circulating progenitor cells (Han *et al.*, 2001; Sata *et al.*, 2002) - a possibility considered in section 1.1.3.5. Nonetheless, reduced lesion size is observed following blockade of proliferative pathways either at the level of cell proliferation, by sirolimus (Burke *et al.*, 1999), paclitaxel (Axel *et al.*, 1997), anti-sense oligonucleotides against proliferating cell nuclear antigen (PCNA) or cyclin-dependent kinase-2 (Morishita *et al.*, 1993; Morishita *et al.*, 1994), or by modifying activity of individual mitogens including PDGF (Banai *et al.*, 1998; Matsuno *et al.*, 2002) and IL-1 (Chamberlain *et al.*, 2006). Migration of medial VSMCs to the intima is also likely to depend on remodelling of the basement membrane and medial ECM. Several relevant enzymes, including MMP-9, are activated in the growing neointima (Godin *et al.*, 2000) and MMP-9 deficiency both impairs VSMC migration *in vitro* and reduces neointima formation *in vivo* (Cho & Reidy, 2002).

Neointimal lesion growth is not directly coupled to VSMC accumulation. Zwolak *et al* (1987), for example, describe lesion growth by continued extracellular matrix deposition in a model of vein graft hyperplasia. Rate of VSMC death may also be important because Clowes *et al* (1983) report high levels of VSMC proliferation in balloon-injured rat carotid arteries, without lesion growth. Indeed, continual VSMC apoptosis occurs in the neointima in several models (Han *et al.*, 1995; Reis *et al.*, 2000) and treatments have been described that appear to modify lesion size by regulation of apoptosis (Blanc-Brude *et al.*, 2002; Sata *et al.*, 2003; Tanja Rauma-Pinola, 2006). Perhaps the greatest discrepancy of kinetics is between animal studies of neointima formation and man. In almost all animal models, neointimal lesion size peaks approximately 1 month after the initiating injury. In man, whilst it is difficult to precisely determine the time course of lesion formation, the incidence of post-PTCA restenosis peaks between 3 and 7 months (Holmes *et al.*, 1984; Nobuyoshi *et al.*, 1988; Serruys *et al.*, 1988; Fitzgibbon *et al.*, 1996). This inconsistency is probably attributable to the state of the vessels in which neointima formation is

elicited since most animal models involve injury of a young, healthy artery whereas, in man, re-vascularisation procedures are performed in aged, atherosclerosis-ridden vessels. Regardless, inhibition of cell proliferation using sirolimus (Moses *et al.*, 2003) or paclitaxel eluting stents (Stone *et al.*, 2004) or local delivery of ionizing radiation (Teirstein *et al.*, 1997; Leon *et al.*, 2001) has proven a successful strategy for the reduction of post-interventional restenosis in human patients.

1.1.3.4 *Role of the endothelium and oxidative stress*

The endothelium releases factors, such as NO and PGI_2, that suppress the inflammatory, proliferative and thrombotic processes implicated in neointima formation. Indeed, a role for these factors is supported by the ability of prostacyclin synthase (Todaka *et al.*, 1999), eNOS gene transfer (Cooney *et al.*, 2007), NO donor drugs (Chaux *et al.*, 1998; Hou *et al.*, 2005) or L-arginine (Schwarzacher *et al.*, 1997; Le Tourneau *et al.*, 1999) to reduce lesion size in several models of endothelium-denuding acute vascular injury. Whether endogenous EC-derived factors modulate neointima formation is more difficult to predict because all common PCIs (Gravanis & Roubin, 1989; Ueda *et al.*, 1991; Komatsu *et al.*, 1998) denude the target vessel of EC. This removal may enhance inflammatory and mitogenic processes and, as such, stimulate lesion growth, but is not sufficient to induce neointima formation (Reidy & Silver, 1985; Miller *et al.*, 2003). After such injury, the endothelium is able to recover by in-growth of cells from the borders of the denuded region and, probably, by recruitment of circulating cells (see section 1.1.3.5). Whether this neo-endothelium is functionally normal is not clear and alterations to morphology and endothelium-dependent responses of varying degrees have been reported (Schatz *et al.*, 1987; Shimokawa *et al.*, 1987; Shimokawa *et al.*, 1989; Miller *et al.*, 2003; Liao *et al.*, 2007). Animal studies indicate that EC re-growth can occur remarkably quickly, with complete recovery of a monolayer of cells expressing endothelial markers (von Willebrand factor$^+$, CD31$^+$) and mediating at least some 'endothelium'-dependent vasodilator response within 7-14 days in most models (Fishman *et al.*, 1975; Clowes *et al.*, 1976; Reidy & Silver, 1985; Pellegreffi & Branzoli, 1987; Schatz *et al.*, 1987; Lindner *et al.*, 1989; Miller *et al.*, 2003; Liao *et al.*, 2007). In others, recovery can take up to 6 weeks (Shimokawa *et al.*, 1987),

but only in rare cases is complete recovery not achieved (Haudenschild & Schwartz, 1979; Reidy *et al.*, 1983). In most models, therefore, the speed of endothelial recovery appears to be greater than that of lesion growth, indicating that a substantial window exists in which the endothelium might modulate neointima formation. Further, administration of the EC mitogen, vascular endothelial growth factor (VEGF), accelerates re-endothelialisation of denuded regions and inhibits lesion growth (Asahara *et al.*, 1995; Hiltunen *et al.*, 2000; Hutter *et al.*, 2004).

The effects of endothelium-targeted interventions in animal models of acute vascular injury further suggest this potential. eNOS deficiency, for example, increases lesion size (Moroi *et al.*, 1998; Zhang *et al.*, 2006), but has only so far been investigated in models of neointima formation that do not involve endothelial denudation. Chronic administration of the NOS inhibitor, L-NAME, however, has been shown to exacerbate lesion formation, even in EC-denuding models of injury (Le Tourneau *et al.*, 1999). Treatments that might be expected to improve endothelial function have also been investigated. A variety of anti-oxidants including probucol (Ferns *et al.*, 1992; Ishizaka *et al.*, 1995; Miyauchi *et al.*, 1998; Yokoyama *et al.*, 2004), N-acetyl cysteine (Hayashi *et al.*, 2001) and even red wine (Feng *et al.*, 1999) reduce neointima formation in EC-denuding models of vascular injury. This indicates that production of oxidative species may contribute to neointima formation, probably, in part, by reducing the bioavailability of NO. Dysfunctional ECs, as perhaps may be present early after re-endothelialisation, and other neointimal cell types (VSMCs, macrophages) are certainly capable of producing oxidative species. Indeed, in hph-1 mice (a model of NOS uncoupling and EC dysfunction secondary to tetrahydrobiopterin (BH_4) deficiency), neointima size after EC-denuding injury is increased (Wang *et al.*, 2005a). Complementary data from Liao *et al* (2007) indicate that lesion size can be reduced, and basal NO release increased, by improving NOS function selectively in EC, by EC-specific over-expression of GTP cyclohydrolase I. These studies further suggest that EC can regulate lesion development, despite its removal by vascular injury and subsequent re-growth, and implicate the balance between production and oxidative deactivation of endothelium-derived NO in the pathogenesis of this process. It is also important to note that expression of NOS

isoforms has been described in VSMCs of the developing neointima and, thus, that beneficial mediators (such as NO) normally produced by the endothelium may be released from other cells in the lesion (Arthur *et al.*, 1997; Wang *et al.*, 2005a).

Without longitudinal studies, the time-course of post-interventional re-endothelialisation is difficult to determine in man. Necropsy samples of balloon-injured coronary arteries have been reported to possess a complete EC-like monolayer as soon as 1 month after intervention (Gravanis & Roubin, 1989). Samples obtained 5 and 12 months after injury also possess an intact endothelium (Ueda *et al.*, 1991), and at 6 months endothelium-dependent vasomotion of these injured vessels is present (Sabate *et al.*, 1999). After stent implantation, Schatz *et al* (1991) report endothelial recovery at 2 months, whilst Komatsu *et al* (1998) describe partial EC coverage in a vessel 9 weeks after intervention. As in animal studies, therefore, recovery of the endothelium typically occurs prior to the peak incidence of restenosis (3-7 months), with the exact kinetics probably dependent on the nature of injury and underlying pathology. The presence of existing endothelial dysfunction, implicit in the atherogenic process, may also modulate the ability of EC to regulate neointima formation. Indeed, several studies indicate that peripheral flow-mediated dilatation is an independent predictor of the incidence of restenosis in coronary arteries (Kitta *et al.*, 2005; Patti *et al.*, 2005). This may, however, simply reflect the presence of other influences that contribute to both restenosis and EC dysfunction. Evidence from endothelium-targeted interventions in man is also limited. VEGF gene transfer has been suggested to be ineffective at reducing in-stent restenosis, but only in a small trial of low-risk patients of whom just 6 developed restenosis (Hedman *et al.*, 2003). More encouragingly, probucol and its derivative AGI-1067 are effective at reducing the incidence of restenosis (Setsuda *et al.*, 1993; Tardif *et al.*, 1997; Yokoi *et al.*, 1997), an effect animal studies attribute to its potent anti-oxidant potential rather than lipid lowering action (Ferns *et al.*, 1992).

1.1.3.5 *Role of circulating progenitors*

The traditional model of neointima formation proposes that VSMC accumulation and EC re-growth are the result of proliferation of local vascular cells (Spaet *et al.*, 1975;

Stemerman *et al.*, 1977; Haudenschild & Schwartz, 1979; Lee *et al.*, 1993). More recent evidence indicates that bone marrow-derived circulating progenitor cells can adopt VSMC or EC-like phenotypes to participate in the formation of a neointima after vascular injury. Numerous studies in which the bone marrow of myelo-ablated mice is reconstituted with that of transgenic reporter animals demonstrate that 20-70% of the neointimal cells that express VSMC markers (αSMA, calponin), possess the donor genotype (Han *et al.*, 2001; Saiura *et al.*, 2001; Sata *et al.*, 2002; Tanaka *et al.*, 2003; Wang *et al.*, 2006; Tanaka *et al.*, 2008). Similarly, between 10-50% of the ECs regenerated after denudation, appear to be donor-derived (Sata *et al.*, 2002; Werner *et al.*, 2002; Tanaka *et al.*, 2003). Moreover, specific populations of progenitor cells are implicated with mesenchymal stem cells contributing most (Wang *et al.*, 2008). The importance of progenitor cell recruitment in neointima formation is not uniform between models, however, and may be related to the degree of medial damage inflicted by the initial injury (Tanaka *et al.*, 2003).

1.1.4 *Modelling the response to vascular injury*

Direct study of human disease is the obvious means from which to gather information regarding pathogenesis and potential therapies must, of course, ultimately be tested in man. The practical and ethical limitations of working with human tissue, however, have necessitated the development of many animal models of neointima formation, from which much of our understanding of the mechanisms of this process have been gained. Vascular damage is typically induced by mechanical injury but a diverse range of other insults to the vasculature have also been devised that result in rapid development of a fibro-proliferative neointima.

1.1.4.1 *Intra-luminal injury models*

Models of mechanical damage, inflicted from within the vascular lumen, directly mimic the nature of injury induced by PCIs. Much of the early work on the pathogenesis of neointima formation was performed using intra-luminal balloon injured rat internal carotid arteries. The degree of injury is adjustable by altering inflation pressure and balloon compliance but typically induces rapid and

reproducible formation of a moderate neointima (intima/media ratio ~1-1.5; Clowes & Karnowsky, 1977; Fingerle *et al.*, 1990; Morishita *et al.*, 1993; Morishita *et al.*, 1994; Mnjoyan *et al.*, 2008). Balloon-injury and stent implantation are also used to elicit neointima formation in larger animals, particularly swine (Steele *et al.*, 1985; Heras *et al.*, 1989). These species carry the considerable advantage that clinical interventional devices can be directly employed, but to ensure severe and reproducible injury, balloons and stents that would be considered over-sized for a human artery of the same calibre are commonly used (Schwartz *et al.*, 1992b; Burke *et al.*, 1997; Touchard & Schwartz, 2006). Nonetheless, the neointimal lesions-induced by porcine coronary artery injury are characterised by their similarity to those in man and, these models have the highest accuracy in predicting the clinical efficacy of anti-re-stenotic treatments (Schwartz *et al.*, 1992b; Schwartz *et al.*, 1994; Touchard & Schwartz, 2006).

Intra-luminal injury to the murine vasculature presents a particular challenge due to its scale. Lindner *et al* (1993) first reported a wire-injury model, which entails insertion of an angioplasty guide wire, of comparable diameter to the vessel lumen, into the mouse carotid artery. The neointimal response, however, is modest (intima/media ratios less than 0.1 have been reported), strongly dependent on mouse strain, and is of variable reproducibility (Lindner *et al.*, 1993; Sullivan *et al.*, 1995; Iafrati *et al.*, 1997; Xu, 2004; Liao *et al.*, 2007). This model was adapted by two separate groups for application to the femoral artery and these derivatives have been widely adopted. Both elicit a similar neointimal response, and differ only in that Sata *et al* (2000) describe insertion of the wire via the popliteal branch rather than the femoral artery itself (Roque *et al.*, 2000); thereby allowing blood flow to be preserved over the injured region. Femoral artery wire-injury addresses each of the short-comings of the carotid wire-injury model - eliciting reproducible formation of a large VSMC-rich neointimal lesion (intima/media ratios ~2) in a variety of mouse strains (Roque *et al.*, 2000; Sata *et al.*, 2000): a greater responsiveness presumably attributable to the use of a wire of the same diameter in a substantially smaller vessel (Roque *et al.*, 2000; Kawasaki *et al.*, 2001). Wire-insertion is associated with severe medial stretch damage and endothelial denudation (Roque *et al.*, 2000; Sata *et al.*,

2000). Within hours of injury, apoptosis in the media and adhesion of platelets and neutrophils to the luminal surface occurs, and within 7 days macrophage infiltration is present (Reis *et al.*, 2000; Roque *et al.*, 2000; Sata *et al.*, 2000; Osaka *et al.*, 2007). Neointima formation is apparent from 7 days and reaches a maximum size between 21 and 28 days where it is stable for at least 56 days (Roque *et al.*, 2000; Sata *et al.*, 2000). Re-growth of the endothelium is also complete by 28 days (Sata *et al.*, 2000), and unpublished data from this laboratory indicates this occurs between 7 and 14 days (Macdonald *et al.,* Unpublished data). Indeed, data from the carotid wire-injury model suggests restoration of endothelium-dependent vasodilatation occurs by 10 days (Liao *et al.*, 2007). Proliferation in both the media and neointima peak at 14 days (Reis *et al.*, 2000) and a substantial role for bone marrow-derived VSMCs and ECs is indicated (Saiura *et al.*, 2001; Tanaka *et al.*, 2003; Shoji *et al.*, 2004).

1.1.4.2 *Peri-vascular injury models*

The relative complexities of these mechanical modes of injury, particularly in the mouse, have led to the adoption of numerous neointima-inducing insults that can be applied from the adventitial surface of the vascular wall. Robert Buck (1961) originally described ligation-induced neointima formation in the rat carotid artery but this mode of injury only found common use after its adaption to the mouse (Kumar & Lindner, 1997). Permanent ligation of the common carotid artery abolishes blood flow and elicits formation of a moderate, muscular neointima (intima/media ratio ~1-1.5) within 28 days without overt EC damage (Kumar *et al.*, 1997; Kumar & Lindner, 1997; Godin *et al.*, 2000). Between 7 and 14 days, intimal and medial proliferation rates peak and formation of platelet-rich thrombus in the vessel lumen is apparent (Kumar & Lindner, 1997; Godin *et al.*, 2000). Leucocytes adhering to the endothelium are detectable from 1 day after ligation, and at later time-points are associated with platelet aggregates and the adventitia (Godin *et al.*, 2000). Little role for VSMC-like cells derived from circulating progenitors is apparent in this model, presumably due to the absence of direct injury to media, from where these cells may also be derived (Tanaka *et al.*, 2003). Numerous potential stimuli exist for inflammation and neointima formation following arterial ligation. Loss of blood flow and, therefore, low shear stress may promote EC dysfunction and adhesion molecule

expression (Kumar & Lindner, 1997; Godin *et al.*, 2000). Indeed, endothelial ICAM-1 and VCAM-1 immunoreactivity can be detected from 3 days in this model (Kawashima *et al.*, 2001). The absence of blood flow may also result in hypoxic damage to vascular wall cells and formation of stasis thrombus in the lumen, containing activated platelets that present leucocyte adhesion molecules and release mitogens (Kumar & Lindner, 1997; Godin *et al.*, 2000).

An alternative 'endothelium-intact' model of neointima formation comprises installation of a loose-fitting sheath of polyethylene or silicone tubing around an isolated artery. This peri-vascular cuff model was first applied to the rabbit carotid artery (Booth *et al.*, 1989), and later adapted to the mouse femoral artery (Moroi *et al.*, 1998). Over 14 days, a small neointima (intima/media ratios 0.5-1) reliably develops in the cuffed portion of vessel without thrombus formation or disruption of the endothelium (Kockx *et al.*, 1992; Kockx *et al.*, 1993; Moroi *et al.*, 1998). Although various explanations for this response have been suggested (cuff toxicity and inflammatory activation, vessel distortion, damage to the adventitial vaso vasorum, lymphatic vessels or innervation) the exact stimuli for neointima formation remain unclear (Kockx *et al.*, 1992). Electrical injury to the arterial wall is a more overt stimulus for neointima formation. Injury to the mouse femoral artery by a single application of electrical current across the adventitial surface results in necrosis of medial VSMCs, destruction of the endothelium and transient mural thrombus formation. Intimal proliferation peaks between 7 and 14 days, and results in formation of a moderate neointima (intima/media ratios ~1.5; Carmeliet *et al.*, 1997; Carmeliet *et al.*, 1998).

1.2 ENDOTHELIN & ENDOTHELIN RECEPTORS

ET-1 is one of many mediators implicated in pathogenesis of neointima formation, and indeed, a variety of other cardiovascular diseases. This potent vasoconstrictor peptide was first isolated from cultured porcine ECs by Hickey *et al* in 1985. The structure, generation and cardiovascular effects of ET-1 were subsequently described in a single remarkable paper (Yanagisawa *et al.*, 1988). It is now established that ET-1 is the principal cardiovascular isoform of a family of 21 amino acid peptides

comprising ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989; Bloch *et al.*, 1991). ETs possess striking sequence homology with 4 further peptides, sarafotoxins, extracted from the venom of *Atractaspis engaddensis* (Kloog *et al.*, 1988), which also contain the two disulphide bridges (**Figure 1-2**) that give these peptides their characteristic 'open loop' tertiary structure (Janes *et al.*, 1994).

1.2.1 *Synthesis and release of endothelins*

ET-1 is generated from precursor peptides via a two-step proteolytic pathway (**Figure 1-2**). Transcription of a gene on chromosome 6 generates mRNA encoding the 212 amino acid peptide, preproET-1 (ppET-1), which, once translated, is stripped of its signal sequence and secreted into the cytoplasm as proET-1 (Inoue *et al.*, 1989). ProET-1 is further cleaved by a furin-like endopeptidase to the 38 amino acid precursor big ET-1 which circulates in plasma at low concentration but is not thought to possess significant bioactivity (Yanagisawa *et al.*, 1988; Kimura *et al.*, 1989; Denault *et al.*, 1995). Removal of a further 17 C-terminal residues classically (but not exclusively) by ET-converting enzymes (ECE) produces the mature 21 amino acid ET-1 (Yanagisawa *et al.*, 1988; Ohnaka *et al.*, 1990; Opgenorth *et al.*, 1992).

In humans and rodents two distinct isozymes of ECE, ECE1 (Xu *et al.*, 1994) and ECE2 (Emoto & Yanagisawa, 1995), catalyse the second (rate-limiting) step in ET-1 synthesis (Opgenorth *et al.*, 1992). These type II integral membrane proteins have catalytic domains characteristic of zinc metalloproteinases and are closely related to other enzymes of this class, (Xu *et al.*, 1994). ECE1 has four functionally-similar isoforms (ECE1a-d), defined by slight variations in NH_2-terminal sequence, which determine their discrete sub-cellular localisation to plasma and intracellular membranes (Russell & Davenport, 1999). ECE2 has at least four distinct isoforms but is less well characterised than ECE1 and may have a role in the processing of big ET-1 during its transit through intra-cellular secretory pathways (Ikeda *et al.*, 2002).

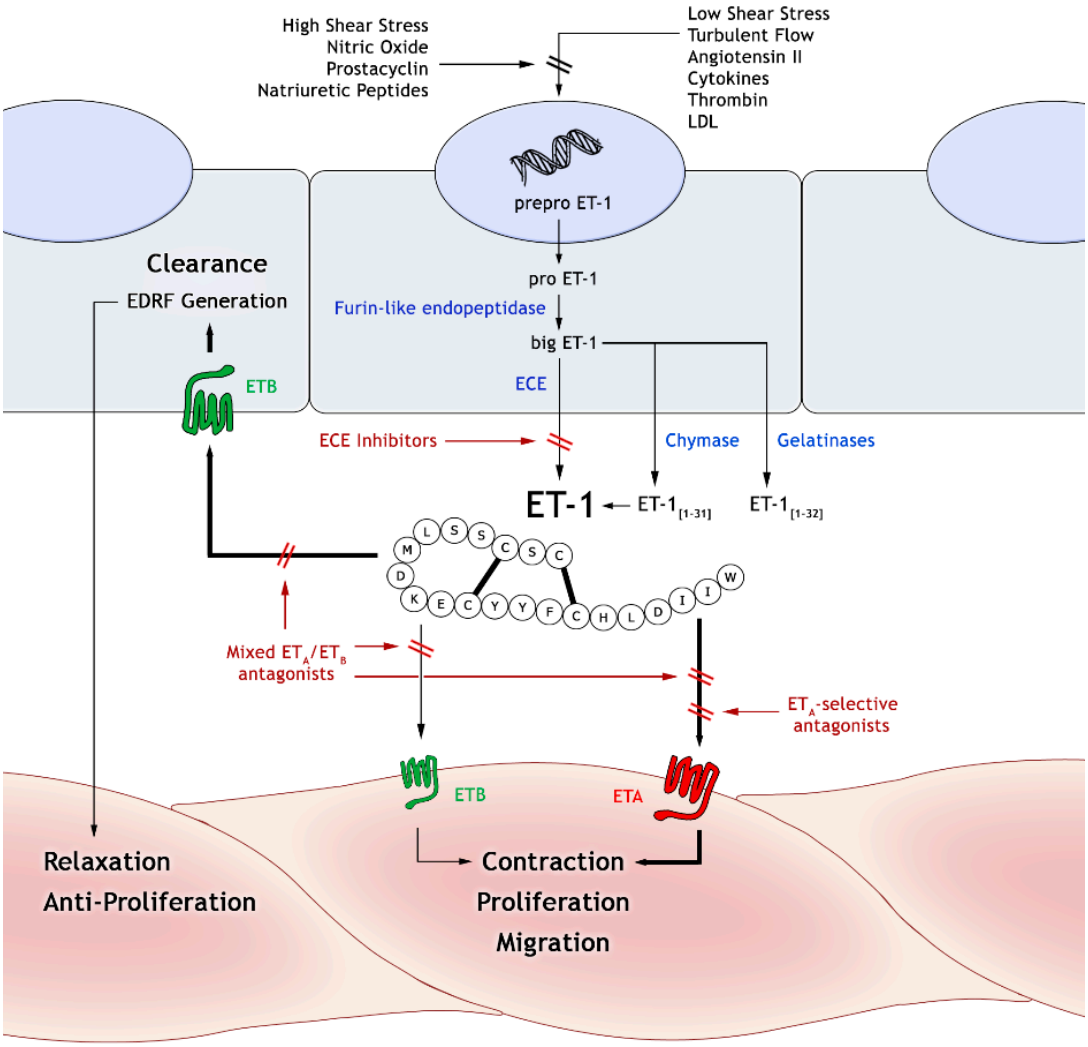


Figure 1-2 Generation and action of ET-1 within the vascular wall.

The 21 amino acid peptide, ET-1, is the eventual product of a gene on chromosome 6 which encodes preproET-1 protein. This is converted to proET-1 on secretion into the cytoplasm which itself undergoes enzymatic cleavage by a furin-like endopeptidase to form bigET-1. ET-1 is generated from bigET-1 by ECEs and is excreted predominantly abluminally into the vessel wall. Other enzymes may also process big ET-1 to yield bioactive 31- and 32-amino acid isoforms. ET-1 stimulates contraction, proliferation and migration of vascular cells by a direct action on VSMC ETA, and to a lesser extent, VSMC ETB receptors. ET-1 promotes ET-1 clearance and indirect relaxation and anti-proliferation by stimulation of the EC ETB receptor. The identity of the vasodilators released from the endothelium following ET_B receptor stimulation varies between vascular beds. The principal pharmacological approaches to modulating the action of ET-1 are (1) inhibition of synthesis with ECE inhibitors and (2) blockade of receptors using ETA selective or non-selective ETA/B antagonists. EDRF, endothelium-derived relaxing factors; ECE, endothelin-converting enzyme; VSMC, vascular smooth muscle cell; EC, endothelial cell. Reproduced from Kirkby *et al* (2008).

Surprisingly, ECE1/ECE2 double knockout mice continue to produce significant amounts of ET-1 (Yanagisawa *et al.*, 2000), albeit alongside developmental defects characteristic of inhibition of the ET axis, suggesting substantial redundancy of this pathway. Indeed, several other enzymes can cleave big ET-1 to form both ET-1 and isoforms bearing extended C-terminal sequences. These include chymase (Wypij *et al.*, 1992) and gelatinolytic MMPs (Fernandez-Patron *et al.*, 1999), which mediate the production of $ET\text{-}1_{[1\text{-}31]}$ and $ET\text{-}1_{[1\text{-}32]}$ respectively.

In ECs, ET-1 is released through both constitutive and regulated (rapid release) pathways (Russell & Davenport, 1999). Vesicles originating in the trans-Golgi network and Weibel-Palade bodies display immunoreactivity for ET-1, ECE and big ET-1 suggesting that, in addition to their role in transport and storage, they serve as sites of ECE-regulated ET-1 synthesis (Barnes *et al.*, 1998). ET-1 release via the constitutive pathway is regulated principally at the level of gene transcription. Synthesis is enhanced by protein kinase C (PKC)- and phospholipase C (PLC)-dependent pathways in response to low or turbulent shear stress, hypoxia, cytokines, angiotensin II and catecholamines. In contrast, increased levels of cyclic nucleotide monophosphates associated with high shear stress, NO, PGI_2 and natriuretic peptides suppress ET-1 production (Gray & Webb, 1996). Although EC are the main physiological source of vascular ET-1 in humans and animals (Properzi *et al.*, 1995), it is now recognised that VSMCs, macrophages, leucocytes, cardiomyocytes and fibroblasts are all capable of ET-1 production (Nunez *et al.*, 1990; Resink *et al.*, 1990; Firth & Ratcliffe, 1992). In the kidney, tubular epithelial cells, mesangial cells and podocytes also release ET-1 (Kohan, 1997). Indeed, the kidney contains amongst the highest ET-1 concentrations of any organ. (Morita *et al.*, 1991).

1.2.2 *Clearance of endothelins*

Clearance of ETs from plasma occurs primarily in the lungs (Anggard *et al.*, 1989) by endocytosis and degradation of a complex of the ETB receptor and ET ligand (Fukuroda *et al.*, 1994a; Dupuis *et al.*, 2000). The speed of this clearance, together with the polarised, abluminal nature of ET-1 secretion by ECs, emphasises the poor

predictive value of plasma ET-1 concentration as a measure of synthesis and has resulted in difficulty assessing alterations to ET-1 activity in disease. EC-specific, but not inner-medullary collecting duct (IMCD) cell-specific, deletion of ETB receptors results in a 4-fold increase in plasma [ET-1] and impaired clearance of exogenous ET-1, supporting the view that EC ETB receptors contribute much of the capacity for ET-1 clearance (Bagnall *et al.*, 2006; Ge *et al.*, 2006). Enzymatic degradation also contributes to the clearance of ET-1. Neutral endopeptidase, probably that located in the brush-border vesicles of the proximal tubule, catabolises ET-1 and inhibition of this enzyme increases plasma and urinary ET-1 concentrations (Abassi *et al.*, 1992). Indeed, a significant role for the kidney in the clearance of ET-1 is indicated by the demonstration that bilateral nephrectomy impairs removal of exogenous ET-1 in rats (Shi *et al.*, 1994). Elegant micro-PET studies suggest that renal ET-1 clearance occurs through both receptor-mediated and enzymatic processes and also highlight the liver as a further important site of ET-1 clearance (Johnstrom *et al.*, 2005).

1.2.3 *Endothelin receptors*

The downstream effects of ET-1 are mediated by two distinct receptor subtypes, ETA and ETB, which belong to the rhodopsin-like G-protein-coupled receptor superfamily. cDNAs encoding both receptors were isolated and cloned within two years of the initial characterisation of ET-1 and exhibit 59% sequence homology (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Frelin *et al.*, 1991; Sakamoto *et al.*, 1991). Human ETA and ETB bind ET-1 with equal affinity but ETA possess at least 100-fold less affinity for ET-3 than do ETB receptors (Davenport, 2002). Two additional receptor subtypes identified in *Xenopus laevis,* have no mammalian equivalent (Karne *et al.*, 1993; Kumar *et al.*, 1994). Additional classification of the ETB subtype (particularly prompted by the differential sensitivity of specific ETB-mediated functional responses to the antagonist PD142893) has not been supported by detailed kinetic studies or the effects of ETB gene deletion (Gray & Webb, 1996; Mizuguchi *et al.*, 1997). Furthermore, splice variants reported for both subtypes have not been shown to have functional significance (Davenport, 2002).

ET receptors are expressed in a variety of human tissues. In the vasculature, VSMCs express both ETA and ETB. ECs typically express only ETB (Molenaar *et al.*, 1993) although ETA-like receptors have been reported in the endothelium of the human and rat brain microvasculature (Kawai *et al.*, 1997; Spatz *et al.*, 1997). The relative expression of ETA and ETB by VSMCs is variable but the ETA:ETB ratio is often considered to increase with vessel size (Tschudi & Luscher, 1994; Davenport *et al.*, 1995a). In the human heart, cardiomyocytes and fibroblasts predominantly express ETA, although ETB expression is more abundant in cardiac conducting tissue (Molenaar *et al.*, 1993). ETB receptors are highly expressed in the renal medulla (Karet & Davenport, 1996; Kuc & Davenport, 2004) on both epithelial and endothelial (*vasa recta*) cells, in humans and animals. Renal ETA receptors are less abundant and are predominantly found on the larger renal vessels.

Stimulation of ETA and ETB elicits diverse physiological responses, including: vasoconstriction, mitogenesis, inflammation, hypertrophy and differentiation. The precise response elicited in a particular tissue is dependent on the identity of the receptor expressed and the second messenger pathways activated - which are still the subject of much discussion (Motte *et al.*, 2006). Further complication may result from the formation of ETA/ETB heterodimers giving rise to ligand-dependent differences in functional response and internalisation kinetics (Gregan *et al.*, 2004). Acute stimulation of either ETA or ETB is associated with an increase in intracellular Ca^{2+}. This is composed of an initial rapid, transient phase associated with release from intra-cellular stores mediated by the inositol-1,4,5-trisphosphate/diacylglycerol pathway, followed by a sustained influx of extracellular Ca^{2+} across the sarcolemma. Although free intercellular Ca^{2+} is a key mediator of altered VSMC tone, ET receptors also directly activate phospholipase A_2, modulate activity of adenylate cyclase and, by multiple pathways, promote membrane depolarisation (Motte *et al.*, 2006). Effects of ET-1 on gene transcription are mediated both by PKC-dependent stimulation of the mitogen activated protein kinase (MAPK) pathways and PKC-independent activation of the signal transducers and activators of transcription pathways (Motte *et al.*, 2006).

1.2.4 *Cardiovascular consequences of ET receptor activation*

ETs are commonly defined by their impressive vasomotor effects (Yanagisawa *et al.*, 1988). Indeed, ET-1 remains one of the most potent and characteristically sustained vasoconstrictors yet described. Vascular tone is maintained, in part, by the action of endogenous ET-1, and, consequently, systemic ET receptor blockade in human subjects lowers blood pressure (Haynes *et al.*, 1996). Bolus administration of an ET receptor agonist (especially a selective ETB agonist such as ET-3 or S6c) in whole animals or the human forearm causes transient hypotension/vasodilatation before the onset of vasoconstriction. Blockade of NO or prostacyclin synthesis at least partially prevents this depressor response, and potentiates the pressor phase (de Nucci *et al.*, 1988; Yanagisawa *et al.*, 1988; Whittle *et al.*, 1989; Gardiner *et al.*, 1990; Clozel *et al.*, 1992; Rogerson *et al.*, 1993; Haynes *et al.*, 1995). Moreover, in isolated vascular rings, ETB stimulation also elicits endothelium-dependent vasodilatation (Takayanagi *et al.*, 1991; Clozel *et al.*, 1993), even in the mouse aorta in some reports (Mizuguchi *et al.*, 1997), despite the monophasic (vasoconstrictor) response of this species to an ET-1 bolus (Giller *et al.*, 1997). Thus, the initial ET-1-evoked depressor action, is primarily mediated by the stimulated production of NO and other endothelium-derived relaxing factors by ETB receptors in the endothelium. The vasoconstrictor phase of the response to ET-1 is principally associated with VSMC ETA receptor activation (Ihara *et al.*, 1992; Haynes & Webb, 1994). VSMC ETB receptor-mediated contraction is also well-documented in many vascular beds though probably contributes little to vascular tone. Indeed, selective ETB receptor blockade *increases* peripheral vascular resistance in healthy humans (Strachan *et al.*, 1999) suggesting the balance between vasodilator and vasoconstrictor ETB pathways favours vasodilatation. In pathological conditions, however, it is possible that this balance may be altered (Cardillo *et al.*, 2000; Pernow *et al.*, 2000). These effects of ET-1 on vascular resistance produce predictable reflex changes in heart rate and cardiac output. Direct effects on the heart including positive inotropy, are also reported (Meyer *et al.*, 1996; Burrell *et al.*, 2000). In many studies, however, concomitant effects on coronary blood flow may limit interpretation of such data.

The kidney is also an important target for ET-1. Exogenous ET-1 stimulates regional changes in renal blood flow, typically inducing vasoconstriction in the renal cortex and vasodilatation in the medulla (Rubinstein *et al.*, 1995). Medullary vasodilatation appears to be solely mediated by ETB receptor stimulation whilst effects on the larger renal vessels and glomerular afferent and efferent arterioles are both ETA and ETB receptor-dependent (Dhaun *et al.*, 2006). In mice, at least, IMCD cell-derived ET-1 exerts an autocrine natriuretic and diuretic effect that is at least partially mediated by ETB and inhibition of the epithelial sodium channel. Indeed, IMCD cell-specific deletion of ETB (but neither IMCD cell-specific ETA nor EC-specific ETB knockout) results in salt-sensitive hypertension (Bagnall *et al.*, 2006; Ge *et al.*, 2006) suggesting that IMCD-derived ET-1 stimulates natriuresis by an autocrine action mediated by ETB.

1.2.5 *Lessons from genetic manipulation of the ET system*

The elegant dissection of the natriuretic response to ET-1 is one example of the power that studies of genetic manipulation of components of the ET system have provided. In particular, such use of conditional gene targeting technology has allowed the analysis of ET receptor function in a cell-specific manner, a goal that is not achievable by pharmacological means and is of great importance given the physiologically antagonistic effects of ET receptors expressed by different cell types. Gene over-expression and non-cell specific knockout studies have also been widely utilized to improve our understanding of the ET system.

1.2.5.1 *Gene over-expression*

Transgenic ET-1 mice, in which the human ppET-1 gene is introduced into the mouse germline under control of its natural promoter, exhibit modestly elevated plasma and renal ET-1 levels, but are not overtly hypertensive (Hocher *et al.*, 1997; Shindo *et al.*, 2002). Cross of ET-1 transgenic mice with $eNOS^{-/-}$ or $iNOS^{-/-}$ mice results in a relative increase in blood pressure, suggesting that vascular or renal NO production may suppress a hypertensive effect of ET-1 in this model (Hocher *et al.*, 2004; Quaschning *et al.*, 2007; Quaschning *et al.*, 2008). Transient viral transfection

of a cytomegalovirus promoter-controlled ppET-1 gene construct in rats, in contrast, results in large increases in both blood pressure and plasma ET-1 (Telemaque-Potts *et al.*, 2000; Telemaque-Potts *et al.*, 2002). The effect of ET-1 gene expression using cell-specific promoters has also been explored. EC-specific ET-1 over-expression using the Tie2 promoter is associated with EC dysfunction, small artery hypertrophy and macrophage infiltration into the vascular wall (Amiri *et al.*, 2004; Amiri *et al.*, 2008). These mice too are normotensive; probably because the vasoconstrictor response to ET-1 is blunted (Amiri *et al.*, 2004). Inflammatory activation is also apparent in cardiomyocyte-specific ET-1 over-expressing mice and this is associated with cardiac hypertrophy and premature death (Yang *et al.*, 2004).

1.2.5.2 *Non-specific gene deletion*

Genetic deficiency of any major component of the ET system including ppET-1, ppET-3, ECE-1, ETA and ETB results in pre- or peri-natal mortality, thereby complicating the study of cardiovascular phenotype. Disruption of the ECE-1/ET-1/ETA axis is associated with abnormal development of cardiac and cephalic neural crest-derived tissues leading to cardiac outflow tract and cranio-facial defects similar to those seen in velocardiofacial syndrome in man (Arinami *et al.*, 1991; Hosoda *et al.*, 1992; Scambler *et al.*, 1992; Kurihara *et al.*, 1994; Valdenaire *et al.*, 1995; Clouthier *et al.*, 1998; Yanagisawa *et al.*, 1998). Disruption of the ECE-1/ET-3/ETB axis results in defects to trunk and enteric neural crest-derived tissues and this is associated with an absence of epidermal melanocytes and aganglionic megacolon. Mutations to the genes encoding these proteins have been described in patients with Hirschsprung's disease and type IV Waardenburg's syndrome; human conditions with similar presentation (Baynash *et al.*, 1994; Hosoda *et al.*, 1994; Yanagisawa *et al.*, 1998). As a result, various 'partial' deletion strategies have been employed to investigate the roles of these gene products, generating some surprising data. For example, heterozygous deletion of the ppET-1 gene increases, rather than reduces, blood pressure (Kurihara *et al.*, 1994), highlighting the role for ET-1 in the central regulation of sympathetic drive (Ling *et al.*, 1998), whilst heterozygous ETA receptor knockout mice are normotensive (Berthiaume *et al.*, 2000a; Berthiaume *et al.*, 2000b). ETB receptor deficiency has been the subject of greater study; in part,

due to the description of a hypomorphic ETB allele which allows the responses to 1/8th normal ETB expression to be investigated (Ohuchi *et al.*, 1999), and the development of rescued ETB-deficient rodents in which neurocristopathies are prevented by introduction of a dopamine-β-hydroxylase promoter-driven ETB transgene (Gariepy *et al.*, 1998). These models of ETB deficiency (summarised in **Table 1-1**) exhibit elevated blood pressure, which is sensitive to dietary salt intake and amiloride treatment. Kidney cross-transplantation studies in rats indicate the importance of both renal and extra-renal ETB genotype in this hypertensive effect, and in some reports it is abolished by concurrent ETA blockade (Ohuchi *et al.*, 1999; Berthiaume *et al.*, 2000a; Berthiaume *et al.*, 2000b; Murakoshi *et al.*, 2002; Elmarakby *et al.*, 2004; Ohkita *et al.*, 2005; Fryer *et al.*, 2006). Loss of ETB expression is also associated with elevated plasma ET-1 levels (or impaired ET-1 clearance), down-regulation of ETA receptor expression, and EC dysfunction secondary to oxidative stress (Berthiaume *et al.*, 2000a; Berthiaume *et al.*, 2000b; Gariepy *et al.*, 2000; Quaschning *et al.*, 2005).

1.2.5.3 *Cell-specific gene deletion*

Conditional gene targeting using the Cre-*loxP* recombination system offers considerable advantages to the study of the ET system because inducible or cell-specific control of expression can avoid the developmental phenotype resulting from deficiency in the ET system. Moreover, cell-specific gene deletion studies can determine the contribution of ET and its receptors in specific cell types to the function of complex tissues such as the vascular wall and kidney, where the activity of the ET system is a composite response of diverse actions in many cell types. This is achieved by the introduction of two loxP sequences, flanking a critical portion of the gene of interest, by homologous recombination. These animals are subsequently crossed with mice bearing a transgenic insertion of a construct encoding the bacteriophage enzyme Cre recombinase. Deletion (or rather down-regulation) of the targeted gene occurs because Cre mediates the excision of loxP-flanked ('floxed') sequences by recombination, and this can be controlled in a spatial and/or temporal manner using specific promoters for Cre expression.

Model	Species	Blood Pressure	Plasma ET-1	Endothelial function	ETA expression	Reference(s)
ETB -/-, neonatal	Mouse				Reduced lung and brain ETA ligand binding	(Davenport & Kuc, 2004) (Kuc *et al.*, 2006)
ETB -/-, rescued	Mouse	Increased (salt-sensitive)		Impaired aortic EC-dependent vasodilatation	Reduced ETA-mediated aortic constriction	(Murakoshi *et al.*, 2002) (Quaschning *et al.*, 2005)
ETB -/-, rescued	Rat	Increased (salt-sensitive)	Increased	Increased aortic superoxide production	Reduced renal ETA ligand binding	(Gariepy *et al.*, 2000) (Taylor *et al.*, 2003) (Elmarakby *et al.*, 2004)
ETB -/s	Mouse	Increased	Unchanged compared to +/s			(Ohuchi *et al.*, 1999)
ETB +/s	Mouse	Unchanged				(Ohuchi *et al.*, 1999)
ETB +/-	Mouse	Increased	Impaired clearance of [^{125}I]-ET-1		Reduced hypotension to ETA blockade	(Berthiaume *et al.*, 2000b)
EC-specific ETB -/-	Mouse	Unchanged	Increased; Impaired clearance of [^{125}I]-ET-1	Impaired aortic EC-dependent vasodilatation	Unchanged	(Bagnall *et al.*, 2006) (Kelland *et al.* Unpublished data)
IMCD-specific ETB -/-	Mouse	Increased (salt-sensitive)	Unchanged			(Ge *et al.*, 2006)

Table 1-1 Phenotypic changes in models of ETB receptor deficiency.

Non-specific ETB deficiency is, in most reports, associated with increased blood pressure which is sensitive to dietary salt intake, and reductions in ETA receptor function or ligand binding. Elevated plasma ET-1 or reduced ET-1 clearance, and impaired aortic endothelial dysfunction also result from loss of ETB. Specific deletion of ETB from IMCD cells results in elevated blood pressure and salt-sensitivity without increasing plasma ET-1, whilst EC-specific ETB knockout impairs ET-1 clearance and aortic endothelium-dependent vasodilatation without altering blood pressure. ETB -/s and ETB +/s, refer to animals bearing a hypomorphic ETB allele, and respectively possess 1/8th and 5/8th normal ETB receptor expression levels. ETB -/-, rescued refers to animals possessing a dopamine-β-hydroxylase ETB transgene in addition to ETB deletion. EC, endothelial cell; IMCD, inner medullary collecting duct epithelial cell.

Mice bearing floxed portions of the genes encoding ET-1, ETA and ETB have so far been used for investigations into the roles of ET and its receptors in cardiomyocytes, ICMD cells, neurons and ECs. In the heart, cardiomyocyte-derived ET-1 appears to contribute to the pathogenesis of several cardiomyopathies (Huang *et al.*, 2002; Shohet *et al.*, 2004) whilst, in contrast, cardiomyocyte-specific ETA receptor deficiency does not alter cardiac function or hypertrophic responses (Kedzierski *et al.*, 2003). In the kidney, as discussed above, IMCD-derived ET-1 regulates salt/water homeostasis and, consequently, IMCD ET-1 knockout mice exhibit salt-sensitive hypertension (Ahn *et al.*, 2004), which is partially recapitulated in IMCD ETB knockout mice (Ge *et al.*, 2006).

1.2.5.3.1 EC-specific ETB knockout mice

EC-specific ETB knockout mice were produced, in part, to determine whether a paracrine action of IMCD-derived ET-1 on *vasa recta* ECs contributes to the natriuretic response. These animals were produced by the crossing floxed ETB mice with the Tie2-Cre transgenic mice produced by Kisanuki *et al* (2001). Tie2-Cre mice, when crossed with reporter strains, exhibit strong pan-endothelial expression of Cre with additional recombination apparent in the mesenchymal cells of the atrioventricular canal and proximal cardiac outflow tract (Kisanuki *et al.*, 2001). This Cre construct has been shown to mediate ~80% deletion of the targeted gene in lung ECs isolated from EC-specific connexin 43 knockout mice, whilst expression in brain grey matter is not altered (Liao *et al.*, 2001).

EC-specific ETB knockout mice have been validated using several techniques. Binding of [^{125}I]-ET-1 to EC-enriched lung homogenates is substantially reduced by EC-specific ETB deficiency, and that which remains is almost abolished by pre-incubation with an ETA antagonist. Similarly, densitometry measurement of whole body autoradiographs of binding of an ETB-selective radioligand ([^{125}I]-BQ3020) indicates dramatic reductions in EC-rich tissue (>94% in lung parenchyma and >96% in the renal medulla). In contrast, in non-EC ETB rich areas such as the respiratory bronchi, no significant alteration is apparent (Kelland *et al.,* Unpublished data). ETB deficiency also impairs ETB agonist-stimulated vasodilatation in aortic rings to a

similar extent as pre-incubation with an ETB antagonist (Bagnall *et al.*, 2006). EC-specific ETB-deficient mice are normotensive and do not exhibit an exaggerated blood pressure response to increasing dietary salt intake; suggesting that IMCD-derived ET-1 does not regulate natriuresis by paracrine activation of *vasa recta* EC ETB receptors (Bagnall *et al.*, 2006). This also indicates that systemic, ETB-mediated, EC-dependent vasodilatation is not critical to the regulation of blood pressure in the mouse, despite modest impairment of acetylcholine-stimulated EC-dependent vasodilatation. A 4-fold elevation in plasma ET-1 concentration is apparent in these mice (Bagnall *et al.*, 2006) and this is accompanied by impaired clearance of [^{125}I]-ET-1 of similar degree to that seen following ETB antagonist administration, indicating that ETB expressed by EC is the primary mechanism for the clearance of ET-1 from plasma (Kelland *et al.* Unpublished data). Finally, following exposure to hypoxic conditions, EC-specific ETB deficiency is associated with an exaggerated increase in right ventricular pressures and hypertrophic remodelling of pulmonary arteries, indicating that EC ETB may exert a protective role in pulmonary hypertension (Kelland *et al.* Unpublished data).

1.2.6 *Pharmacological tools for manipulating the ET system*

Excellent pharmacological tools have also been developed for manipulating the ET system, primarily in response to their therapeutic potential. Analogous to inhibition of the RAAS with ACE inhibitors and AT1 receptor antagonists, pharmacological blockade of endogenous ET system has centered on two main strategies: (1) inhibition of ET biosynthesis by ECE and (2) direct antagonism of ET receptors.

1.2.6.1 *ECE Inhibition*

Blockade of ET synthesis, by inhibition of ECE1 (**Figure 1-2**), is an intuitive approach to reducing the activity of ET-1. The effectiveness of this strategy is illustrated by the ability of phosphoramidon to cause vasodilatation and attenuate big ET-1-induced vasoconstriction in healthy volunteers (Haynes & Webb, 1994). Many studies have also shown potentially beneficial effects of ECE inhibition in disease models (reviewed by Jeng *et al.*, 2002). ECE inhibitors, however, have been eclipsed

by ET receptor antagonists both in experimental and in clinical use. This limited enthusiasm for ECE inhibition may be due to theoretical concerns regarding the approach; namely, the redundancy in ET-1-generating pathways and the presence of non-ECE big ET-1-processing enzymes. In addition, should ECE inhibitors successfully reduce ET-1, they would (as with mixed ETA/B receptor antagonists) reduce the beneficial ETB-receptor-mediated actions (vasodilatation and natriuresis) of endogenous ET-1, with the sole advantage that they would preserve receptor-mediated ET-1 clearance.

1.2.6.2 *ET receptor antagonists*

Non-peptidic and peptidic ET receptor antagonists, developed from diverse sources in the wake of the initial characterisation of ET-1, have been pivotal in defining the role of ETs in cardiovascular physiology and pathophysiology (Battistini *et al.*, 2006). Animal and human studies using these compounds demonstrated the ability of ET receptor antagonists to oppose the biological effects of endogenous and exogenous ETs and identified distinct roles for ETA and ETB receptors (Ihara *et al.*, 1992; Haynes & Webb, 1994). Indeed, the defining pharmacodynamic variable of ET receptor antagonists is that of selectivity. To be considered ETA selective, antagonists must display at least 100-fold greater affinity for ETA than for ETB (Davenport, 2002), a selectivity ratio superior to that possessed by many so-called β_1-selective adrenoceptor antagonists. Thus, at optimal concentration, an ETA-selective compound should occupy >90% of ETA receptors while blocking <10% of ETB receptors. At first glance, the argument in favour of selective ETA antagonists seems compelling since ETB receptors mediate beneficial cardiovascular effects (natriuresis, NO release, ET-1 clearance). What remains unclear is whether changes in ETB-mediated response in disease may compromise any net beneficial effect of ETB receptor activation (Cardillo *et al.*, 2000; Pernow *et al.*, 2000). Further, it is suggested that cross-talk between ET receptors may allow the ETB receptor to adopt functions of the ETA receptor under conditions of selective ETA blockade (Fukuroda *et al.*, 1994b; Mickley *et al.*, 1997).

1.2.7 *Clinical progress*

Early clinical trials of the "-sentan" class of drugs have included both selective ETA and mixed ETA/ETB receptor antagonists. The following indications have been studied in large trials: pulmonary arterial hypertension (PAH), scleroderma, heart failure (chronic and acute), essential hypertension, erectile dysfunction and sub-arachnoid haemorrhage (reviewed by Battistini *et al.*, 2006). The results of these studies have been mixed. On one hand, numerous trials of ET blockade in chronic and acute decompensated heart failure have demonstrated, at best, no improvement in survival. In these and other conditions ET antagonists have also been associated with several tolerability issues, most concerningly dose-dependent abnormalities of liver function, in addition to their well-known teratogenicity. On the other hand, the most encouraging outcome of clinical trials has been the approval of bosentan (and, more recently, sitaxsentan and ambrisentan) for treating the symptoms of PAH. Clinical experience gained in this condition has also demonstrated that, with careful monitoring and dose titration, adverse effects of ET blockade are manageable rather than treatment limiting. Further, ongoing phase II and III trials of ET antagonists in a number of other conditions are yielding positive results. These include systemic hypertension (particularly that resistant to existing therapies, or with concurrent kidney disease), prostate cancer, scleroderma and sub-arachnoid haemorrhage. Thus, in the near future, ET antagonists may find use in the treatment of much larger patient populations.

1.3 ENDOTHELINS AS MEDIATORS OF THE VASCULAR RESPONSE TO INJURY

The ability of ET receptor antagonists to moderate processes of neointima formation in man (post-interventional restenosis, vascular graft disease) has yet to be determined by rigorous clinical investigation. This would be of interest not only because ET antagonists may be useful in the primary prevention of these conditions, but also because inhibition of intimal lesion formation may provide a secondary benefit where ET receptor blockade is otherwise indicated, for example, in hypertensive patients. Animal studies strongly suggest a role for ETs in the response to acute vascular injury. ETs stimulate a number of cellular processes crucial to

neointimal lesion formation and consequently, ETA receptor blockade reduces lesion size in many models of acute vascular injury. As in many cardiovascular diseases, however, the role of ETB receptors is less clear because they mediate actions that might either enhance (inflammation, proliferation) or reduce lesion formation (NO release, ET-1 clearance). As such, it is difficult to predict whether selective ETA or non-selective ETA/B antagonist treatment strategies would be more suitable for the prevention of similar vascular remodelling processes in man. This section discusses the current evidence implicating ETs and their receptors in processes of neointima formation.

1.3.1 *Relevant cellular actions of ETs*

1.3.1.1 *VSMC turnover*

The ability of ETs to regulate growth of VSMCs was first reported by Komuro *et al* (1988) in the same year as the isolation and characterization of ET-1 by Yanagisawa *et al* (1988). ET agonists have since been shown to increase DNA synthesis and proliferation in a variety of VSMC lines including human (Yang *et al.*, 1999), bovine (Di Luozzo *et al.*, 2000), rat (Komuro *et al.*, 1988; Hirata *et al.*, 1989; Weissberg *et al.*, 1990; Eguchi *et al.*, 1992; Ohlstein *et al.*, 1992; Mihara & Fujimoto, 1993; Sogabe *et al.*, 1993; Kubo *et al.*, 1998; Daou & Srivastava, 2004) and rabbit aortic VSMCs (Serradeil-Le Gal *et al.*, 1991). Some reports indicate the ETs can induce modest proliferation of quiescent (G_0) cells but, in most, the action of ETs might be better described as co-mitogenic. As such, ETs can elicit more dramatic increases in VSMC proliferation by potentiating the action of other mitogens including PDGF and serum (Hirata *et al.*, 1989; Weissberg *et al.*, 1990; Battistini *et al.*, 1993; Irons *et al.*, 1996). Indeed, ET-mediated mitogenesis in the absence of exogenous growth factors may be a co-mitogenic effect in association with endogenously-produced mitogens (Battistini *et al.*, 1993). *In vivo*, chronic administration of ET-1 produces medial thickening in small mesenteric arteries (Dao *et al.*, 2006), which is attributable to VSMC proliferation (or hypertrophy at lower doses). Given the crucial contribution of VSMC proliferation to neointima formation, the potential for ET-1 to influence lesion formation by stimulation of this response is obvious.

Where investigated, the (co-)mitogenic action of ETs is most commonly sensitive to ETA blockade (Eguchi *et al.*, 1992; Ohlstein *et al.*, 1992; Mihara & Fujimoto, 1993; Sogabe *et al.*, 1993; Kanse *et al.*, 1995; Yoshizumi *et al.*, 1998; Yang *et al.*, 1999) but some studies suggest a role for ETB (Yang *et al.*, 1999; Di Luozzo *et al.*, 2000). This variability probably reflects the origin of the cells investigated: in large arteries such as the aorta (the primary source of VSMC lines for most investigations) the ETA:ETB ratio is typically high (Davenport *et al.*, 1995a; Davenport *et al.*, 1995b). The phenotype of the cells investigated is also likely to be important as VSMCs switch from a contractile to a synthetic phenotype with extended culture. Indeed, Eguchi *et al* (1994) report that in early 'contractile' cultures of rat aortic VSMCs, ET-1 is only weakly mitogenic, and acts via the ETA receptor, whilst in late 'synthetic' cultures, the mitogenic action of ET-1 is dramatically increased, and this becomes sensitive to ETB but not ETA blockade. This issue of phenotype is particularly relevant because proliferating intimal VSMCs in neointima formation are typically of a synthetic phenotype.

ETs may also regulate the content of VSMCs in neointimal lesions by altering cell survival. ET-1 can protect cultured VSMCs from apoptosis induced by various insults, including: NO donors (Shichiri *et al.*, 2000), serum deprivation (Shichiri *et al.*, 2000; Wu-Wong *et al.*, 2000), ϖ-3 fatty acids (Diep *et al.*, 2000) and paclitaxel (Wu-Wong *et al.*, 2000). This effect appears to be mediated by ETA receptor activation (Shichiri *et al.*, 2000), the p42/44 MAPK pathway (Shichiri *et al.*, 2000; Wu-Wong *et al.*, 2000) and inhibition of caspase 3 (Diep *et al.*, 2000). This anti-apoptotic action can also be demonstrated *in vivo* as treatment of deoxycorticosterone acetate (DOCA)/salt hypertensive rats with an ETA antagonist is associated with increased apoptosis of medial VSMCs (Sharifi & Schiffrin, 1997). Thus, endogenous ET-1, acting on the ETA receptor, may prevent apoptosis of intimal VSMCs (a continuous process in the developing neointima) with clear implications for lesion growth. The issue is complicated, however, by a pro-apoptotic action mediated by the ETB receptor. In cultured rat VSMCs exposed to cyclic strain, ET-1 stimulates apoptosis, which is sensitive to ETB blockade or deletion (Cattaruzza *et al.*, 2000). Similarly, in rabbit carotid artery rings subject to excessive

pressure for short periods (160mmHg for 6 hours), ET-1 and ETB expression is increased, and apoptosis of medial VSMC can be prevented by ETB, but not ETA, blockade (Lauth *et al.*, 2000). This pathway may be particularly important in post-interventional restenosis because both PTCA and stent implantation cause stretch-injury to the vascular wall and, in animal models, this is associated with rapid medial apoptosis (Sata *et al.*, 2000). Consequently, ETB receptor stimulation may potentially limit lesion development by reducing the medial VSMC content and/or by increasing death of intimal VSMCs.

1.3.1.2 *Matrix remodelling*

The actions of ETs on fibroblasts are similar to those on VSMCs, stimulating proliferation and migration of Swiss 3T3 cells (Brown & Littlewood, 1989; Takuwa *et al.*, 1989) and of primary fibroblasts derived from several human (Kahaleh, 1991; Cambrey *et al.*, 1994; Shahar *et al.*, 1999; Gallelli *et al.*, 2005) and rat tissues (Yeh *et al.*, 1991; Peacock *et al.*, 1992). The proliferative effect of ETs on fibroblasts is greater in the presence of other mitogens suggesting that, as for VSMCs, they act primarily as co-mitogens (Brown & Littlewood, 1989; Takuwa *et al.*, 1989; Kahaleh, 1991; Yeh *et al.*, 1991). Moreover, ET-1 stimulates the transition of fibroblasts to a myofibroblast phenotype, and increases their production of collagen type I and III as well as connective tissue growth factor release both from fibroblasts and VSMCs (Guarda *et al.*, 1993; Rizvi *et al.*, 1996; Shi-Wen *et al.*, 2001; Shi-Wen *et al.*, 2004; Xu *et al.*, 2004; Rodriguez-Vita *et al.*, 2005; Shi-Wen *et al.*, 2007). These actions may be important in promoting the deposition of matrix in the growing lesion and, thus, contribute to lesion growth. Although in some studies ETA blockade has proven sufficient to inhibit these pro-fibrotic actions of ETs (Shi-Wen *et al.*, 2004; Gallelli *et al.*, 2005), in most they can only be prevented by the combination of ETA and ETB antagonists (Guarda *et al.*, 1993; Cambrey *et al.*, 1994; Xu *et al.*, 1998; Shi-Wen *et al.*, 2001; Clozel & Salloukh, 2005).

The role of ETs in matrix degradation is more complex. On the one hand, ETA activation induces MMP-9 release from neutrophils (which may aid migration of VSMCs from the media by degradation of the elastin-rich elastic laminae) and

amplify local ET levels by generation of ET-$1_{[1-32]}$ (Fernandez-Patron *et al.*, 2001). On the other hand MMP-1 expression in fibroblasts is reduced by ETA activation: an action that may be permissive to ET-induced matrix deposition, as MMP-1 degrades subtypes of collagen that are produced in response to the action of ETs (Shi-Wen *et al.*, 2001).

1.3.1.3 *Inflammation*

ETs have also been implicated as pro-inflammatory mediators, acting directly on polymorphonuclear phagocytes and cells of monocyte lineage. In several studies, ET-1 administration is reported to increase rolling and adhesion/accumulation of neutrophils *in vivo*, an important early process in the response to acute injury (Espinosa *et al.*, 1996; Helset *et al.*, 1996; Boros *et al.*, 1998; Sanz *et al.*, 1999; Sato *et al.*, 2000). Moreover, this has been shown to occur when labelled neutrophils are administered after pre-treatment with ET-1, indicating a direct effect on these cells rather than actions secondary to altered haemodynamics or other indirect actions (Sato *et al.*, 2000). *In vitro* studies suggest that ET-1 increases neutrophil expression of the leucocyte integrin CD11b/CD18 by the p42/44 MAPK pathway and activation of the ETA receptor (Espinosa *et al.*, 1996; Sato *et al.*, 2000; Fernandez-Patron *et al.*, 2001; Jozsef *et al.*, 2002). This is consistent with the demonstration that blockade of ETA or CD18 *in vivo* prevents neutrophil accumulation in response to ET-1 (Espinosa *et al.*, 1996; Boros *et al.*, 1998). ETA receptor activation also increases neutrophil migration *in vitro*, although whether this reflects chemotaxis or chemokinesis is debated (Elferink & de Koster, 1994; Cui *et al.*, 2001). Finally, in addition to recruitment, ET-1 elicits MMP-9 release from neutrophils (Fernandez-Patron *et al.*, 2001) and potentiates stimulated production of O_2^- (Ishida *et al.*, 1990). Thus, ETs, acting primarily on the ETA receptor, might contribute to the recruitment of neutrophils to sites of acute vascular injury and their subsequent activity.

ET receptors are also expressed by monocytes and derived cells. Rat peritoneal macrophages express exclusively ETB whereas human peripheral blood monocytes and THP-1 monocyte-like cells express exclusively ETA. Human peripheral blood monocyte-derived macrophages, express both ETA and ETB (Sakurai-Yamashita *et*

al., 1997; Haug *et al.*, 2001; Grimshaw *et al.*, 2002). ETs appear to promote chemotaxis of both monocytes and macrophages, which, in the latter, is mediated by ETB receptor activation (Achmad & Rao, 1992; Cui *et al.*, 2001; Grimshaw *et al.*, 2002). Monocytes have also been shown to release mediators that are chemotactic and mitogenic both for neutrophils and for macrophages (such as interleukin-8, MCP-1 and M-CSF) in response to ET receptor activation (Helset *et al.*, 1994; Huribal *et al.*, 1994; Cunningham *et al.*, 1997). As with neutrophils, therefore, ETs may be important in the recruitment of macrophages (crucial mediators of neointimal lesion formation) to sites of vascular injury. Further, ETs promote macrophage 'activation' – stimulating Ca^{2+} mobilization and activity of NFκB (Haller *et al.*, 1991; McMillen *et al.*, 1995; Wilson *et al.*, 2001). Accordingly, macrophage ET receptor stimulation increases release of a variety of mediators, including: TNFα, IL-1β, IL-6, IL-8, TXA_2 and O_2^- (Haller *et al.*, 1991; Millul *et al.*, 1991; Helset *et al.*, 1993; McMillen *et al.*, 1995; Chanez *et al.*, 1996; Cunningham *et al.*, 1997; Ruetten & Thiemermann, 1997; Shimada *et al.*, 1998; Speciale *et al.*, 1998; Juergens *et al.*, 2008). Both ETA and ETB have been implicated in mediating such responses, although in most cases, the subtype involved has not been determined (Ruetten & Thiemermann, 1997; Shimada *et al.*, 1998; Speciale *et al.*, 1998; Juergens *et al.*, 2008). ET receptor stimulated release of these mitogenic and pro-inflammatory factors may be important in the developing lesion, sustaining the inflammatory response and contributing to the migration and proliferation of VSMCs.

ETs may further contribute to inflammatory processes by an indirect action on cells of the vascular wall. ET agonists stimulate expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) and cytokines (IL-8) in human cerebromicrovascular and coronary artery ECs (McCarron *et al.*, 1993; Hofman *et al.*, 1998; Fernandez-Patron *et al.*, 2001), presumably by ETB activation. In addition, CRP-induced expression of ICAM-1, VCAM-1 and MCP-1 in human saphenous vein ECs can be prevented by ET receptor blockade (Verma *et al.*, 2002). EC-specific ppET-1 over-expression is associated with MCP-1 expression and macrophage accumulation in the wall of small arteries (Amiri *et al.*, 2008). ETs also regulate inflammatory activation by VSMCs. *In vitro*, in cultured VSMC and *in vivo*,

in DOCA/salt hypertensive rats, ET-1 induces VCAM-1 expression, which can be prevented by anti-oxidants, NADPH oxidase inhibitors or ETA antagonists (Li *et al.*, 2003; Daou & Srivastava, 2004).

1.3.1.4 *Endothelial function*

The vasodilator effects of ETs are well known (see section 1.2.4). This action is mediated by stimulated production of NO and other endothelium-dependent relaxing factors by ETB receptors in endothelial cells and as such, cultured EC from several species demonstrate NO release upon ETB receptor stimulation (Hirata *et al.*, 1993; Tsukahara *et al.*, 1994). Endogenous ETs may, therefore, exert a moderating effect on lesion formation by stimulation of the EC ETB receptor to elicit release of vasodilator factors such as NO and prostacyclin, which are known to inhibit neointimal lesion formation (Moroi *et al.*, 1998; Le Tourneau *et al.*, 1999).

The influence of ET-mediated regulation of endothelial function on neointimal proliferation is more complex, however, because ET receptor activation also stimulates the production of oxidative species. Indeed, in epicardial arteries (Halcox *et al.*, 2001) and the forearm vasculature of human atherosclerotic patients (Bohm *et al.*, 2005), ETA and ETA/B receptor blockade, respectively, improve endothelial function (response to acetylcholine). Also in the human forearm, ET-1 administration impairs EC-dependent vasodilatation and this action can be prevented with ascorbic acid (Bohm *et al.*, 2007). Similarly, ETB-deficient rats (Elmarakby *et al.*, 2004; Quaschning *et al.*, 2005), EC-specific ETB-deficient mice (Bagnall *et al.*, 2006), and EC-specific ET-1 over-expressing mice (Amiri *et al.*, 2004), exhibit impaired EC-dependent vasodilatation, probably owing to the elevated levels of ET-1 in these models. In EC-specific ET-1 over-expressing mice, endothelial dysfunction is inhibited by ascorbic acid and is associated with increased activity of NADPH oxidase and up-regulation of its $gp91^{phox}$ subunit (Amiri *et al.*, 2004). This action has been further investigated *in vitro*: both in cultured VSMC (Wedgwood *et al.*, 2001; Rodriguez-Vita *et al.*, 2005) and ECs (Duerrschmidt *et al.*, 2000; Dong *et al.*, 2005), incubation with ET-1 stimulates O_2^- production and up-regulation of NADPH oxidase subunits ($gp91^{phox}$, $gp47^{phox}$) and this is sensitive to the NADPH oxidase

inhibitor, apocyanin. In VSMCs, this is mediated primarily by the ETA receptor (Wedgwood *et al.*, 2001), whilst in ECs it is (predictably) mediated by ETB (Duerrschmidt *et al.*, 2000; Dong *et al.*, 2005). Apocyanin-sensitive ET-1-induced O_2^- production has also been reported in endothelium-denuded and endothelium-intact rat aortic rings but, in the latter, an additional pathway for O_2^- production appears to be present (Loomis *et al.*, 2005). Here, ET-1 stimulated O_2^- production appears to be mediated by NOS uncoupling, which follows oxidative de-activation of BH_4 by NADPH oxidase-derived O_2^-. Regardless of mechanism, increased ET-1-induced O_2^- production both in ECs and VSMCs may to contribute to lesion formation, because it impairs the bioactivity of NO, induces detrimental signaling pathways in VSMCs leading to mitogenesis and adhesion molecule expression and, at high concentrations, is cytotoxic, potentially amplifying the initial injury.

Another aspect of ET-1-regulated EC function of possible relevance to the pathogenesis of lesion formation is the control of cell turnover. ET-1 and other ET agonists enhance proliferation and migration of isolated ECs from several species and vascular beds, and this is mediated by ETB-stimulated NO release (Goligorsky *et al.*, 1999; Salani *et al.*, 2000; Kuhlmann *et al.*, 2005). In the presence of VSMCs, an additional pathway involving ETA-mediated induction of VEGF expression may also be important (Pedram *et al.*, 1997; Matsuura *et al.*, 1998; Herrmann *et al.*, 2002). Similarly, EC survival is also regulated by ET-1 and apoptosis, induced by either serum deprivation (Shichiri *et al.*, 1997) or homocysteine (Dong *et al.*, 2005), is inhibited by ETB receptor activation. These actions may contribute to re-endothelialisation following mechanical injury and may protect ECs from apoptosis in vascular graft disease where they are subject to damage without denudation.

Finally, ETB receptors expressed by ECs are an important mediator of the clearance of ET-1 from the circulation (see section 1.2.2). Certainly, ETB blockade (Fukuroda *et al.*, 1994a) or EC-specific ETB knockout increases plasma ET-1 concentration (Bagnall *et al.*, 2006), and this may regulate stimulation of non-EC ETB receptors. The extent that ETB receptors on ECs can regulate the levels of ETs in tissue is less clear. Indeed, because ET-1 circulates at such low (picomolar) concentrations, it is

likely that 'tissue' rather than plasma ETs are of greater contribution to intimal lesion formation. Regardless, it is interesting to note that balloon injury results in rapid accumulation of plasma [^{125}I]-ET-1 in the denuded region (Kurata *et al.*, 1995), indicating that ECs do protect sub-endothelial ET receptors from circulating ETs.

1.3.2 *Alterations to expression/function of the ET system*

It is clear that ETs have the potential to regulate many actions that might contribute to neointimal lesion formation. The importance of these effects *in vivo*, however, is dependent on activation of the ET system at sites of lesion formation. As such, many studies have detailed how the ET system is altered in response to vascular injury (Table 1-2). PTCA, but not angiography, in man is associated with a rapid increase in plasma [ET-1] (Ameli *et al.*, 1993; Hojo *et al.*, 2000; Takase *et al.*, 2003). Patients with >50% restenosis continue to have elevated circulating [ET-1] at 3 months, but in those without, it returns to pre-interventional levels. Descriptions of the ETs in tissue from human neointimal lesions are limited. Shirai *et al* (2006) report both ET-1 and ECE immunoreactivity in neointimal VSMCs and macrophages in atherectomy and post-mortem samples of re-stenotic lesions and this is diminished in specimens obtained >3 months after re-vascularisation. Several studies also indicate the presence of ET-1 protein and mRNA in failed saphenous vein and internal thoracic artery bypass grafts (Brody *et al.*, 1996; Masood *et al.*, 1996). These data are supported by results obtained from relevant animal models. Early work in the rabbit carotid balloon injury model indicates ET-1 immunoreactivity is increased within 24 hours of vascular damage (Azuma *et al.*, 1994; Azuma *et al.*, 1995a). Studies of gene expression following balloon injury to the rat carotid artery also indicate rapid induction of the ET system, with ~2-3-fold increases in levels of ppET-1, ppET-3 and ECE-1 mRNA apparent in whole vessels within days, and remaining elevated for several weeks (Wang *et al.*, 1996). In the same model, ECE activity was also increased (~2-fold at 2 weeks) and, in contrast to uninjured vessels, was localised to non-endothelial cell types (Minamino *et al.*, 1997). Similarly, immunohistochemical studies in balloon injury models show ET-1 predominantly localised to the neointima (Azuma *et al.*, 1994; Dashwood *et al.*, 1999), particularly in VSMCs (Wang *et al.*, 1996; Yoshida *et al.*, 2000). ECs do, however, play a role in

Species (Vessel)	Injury	Method of detection	Intimal VSMCs	Medial VSMCs	ECs	Macrophages	Reference
Human (Coronary)	PCIs	IHC	ET-1, ECE, ETA, ETB			ET-1, ECE, ETA, ETB	(Shirai *et al.*, 2006)
Human (Saphenous)	Vein Graft	Autorad		ETA			(Maguire & Davenport, 1999)
Pig (Coronary)	Balloon	Autorad	ET-1, ETA, ETB	ETA, ETB			(Dashwood *et al.*, 1999)
Pig (Femoral)	Cuff	Autorad		ETA/B	ETA/B		(Dashwood *et al.*, 1993)
Pig (Saphenous)	Graft	Autorad			ETA/B		(Dashwood *et al.*, 1993)
Pig (Saphenous)	Graft	Autorad & IHC		ETA	ET-1		(Dashwood *et al.*, 1998)
Rabbit (Carotid)	Balloon	Autorad	ETB *			ETB	(Azuma *et al.*, 1994)
Rabbit (Carotid)	Balloon	Autorad	ETB *	ETA			(Azuma *et al.*, 1995a)
Rat (Carotid)	Balloon	IHC	ET-1				(Wang *et al.*, 1996)
Rat (Carotid)	Balloon	Autorad	ETA *	ETA			(Viswanathan *et al.*, 1997)
Rat (Carotid)	Balloon	ISH	ETA *				(Ferrer *et al.*, 1995)
Rat (Carotid)	Balloon + Diet	ISH	ET-1		ET-1		(Yoshida *et al.*, 2000)

Table 1-2 Distribution of the endothelin system within neointimal lesions.

Studies investigating the localisation of components of the ET system within neointimal lesions have variously indicated the presence of ETs and ET receptors in most major lesion cell types, although the exact distribution is not consistent between reports (except perhaps the predomination of ETA in the media). * ET components are described as localising to the neointima, but not specifically to intimal VSMCs. VSMC, vascular smooth muscle cells; PCI, percutaneous coronary intervention; IHC, immunohistochemistry; Autorad, autoradiography; ISH, *in situ* hybridization; VSMC, vascular smooth muscle cell; EC, endothelial cell.

ET-1 release in the rat carotid artery balloon-injury model after endothelial re-generation (Yoshida *et al.*, 2000), and in a saphenous vein graft culture model where endothelial damage is likely to be less severe (Dashwood *et al.*, 1998).

The changes to ET receptor expression that occur after acute vascular injury are even more dramatic than those to ETs and ECE. In the rat carotid artery, ETA mRNA levels are reported to increase up to 153-fold (peaking at ~3 days) following balloon injury and remain elevated for ~2 weeks (Ferrer *et al.*, 1995; Wang *et al.*, 1996; Viswanathan *et al.*, 1997; Picard *et al.*, 1998). ETA appears principally associated with medial VSMCs (Azuma *et al.*, 1995a; Dashwood *et al.*, 1999), although Dashwood *et al* (1999) also describe strong ETA radioligand binding in the neointima and associated with neutrophils (Dashwood *et al.*, 1998). Changes in ETB expression follow a similar pattern. Large increases (up to 63-fold) in ETB mRNA levels follow rat carotid artery balloon injury, peaking ~2 days post-injury, and declining to a stable, elevated level within 1 week (Wang *et al.*, 1996; Picard *et al.*, 1998). Studies of ETB radioligand binding in the injured rabbit carotid artery also demonstrate increased ETB protein levels post-injury (Azuma *et al.*, 1994; Azuma *et al.*, 1995a). ETB receptors are typically described to predominate in the neointima, associated with intimal VSMCs and macrophages (Azuma *et al.*, 1994; Azuma *et al.*, 1995a; Dashwood *et al.*, 1999). ETB is also reported in the medial VSMC of balloon-injured porcine coronary arteries (Dashwood *et al.*, 1999) and in sites of neo-vascularisation in porcine saphenous vein/carotid artery interposition grafts (Dashwood *et al.*, 1998). Further, binding sites for ET-1, (probably ETB) are present on ECs in the porcine femoral artery cuff model (Dashwood *et al.*, 1993). In man, re-stenotic lesions display strong immunoreactivity for ETA and ETB which is associated with intimal VSMCs and macrophages and declines from 3 months post-injury (Shirai *et al.*, 2006). In contrast, stable saphenous vein grafts harvested from the hearts of transplant recipients have ET receptor expression levels similar to un-injured vessels (Maguire & Davenport, 1999), probably reflecting the graft age. Acute vascular injury, therefore, would appear to cause rapid activation of the ET system with increased ET-1 and ECE expression apparent within 24 hours despite, in most cases, removal of the endothelium. This activation is relatively transient, with

expression levels normalising with a time-course comparable to that of lesion stabilisation, consistent with the self-resolving nature of the response to acute injury. Changes in ET receptor expression following acute vascular injury are even more short-lived, possibly suggesting that the ET system is more important in early (inflammation and medial proliferation) than late (intimal proliferation and matrix deposition) stages of lesion formation.

The activation of the ET system that occurs following acute vascular injury can be considered a response to increased gene expression by resident cells and influx of non-resident cell types that secrete ETs and/or express ET receptors. Both increased expression of ppET-1/ppET-3 and increased activity of ECE-1 are implicated in the response to vascular injury (Minamino *et al.*, 1997; Bohm *et al.*, 2002). Endothelial denudation, as occurs following mechanical injury, results in loss of the suppressive action of NO and PGI_2 on vascular ppET-1 expression (Boulanger & Luscher, 1990) and promotes platelet aggregation and coagulation, stimulating further ET-1 release (Schini *et al.*, 1989; Sethi *et al.*, 2005). Numerous inflammatory cytokines (IL-1, TNFα, MCP-1) and growth factors (fibroblast growth factor-2, TGFβ) that are present in the developing neointima also induce ppET-1, ECE-1 and ET receptor expression (Yoshizumi *et al.*, 1990; Maemura *et al.*, 1992; Cristiani *et al.*, 1994; Woods *et al.*, 1999; Molet *et al.*, 2000). Macrophages too have been shown to release ET-1, and express ET receptors (the subtype of which varies between analysed populations) and may, therefore, directly contribute to lesion ET and ET receptor content (Ehrenreich *et al.*, 1990; Sakurai-Yamashita *et al.*, 1997; Haug *et al.*, 2001; Grimshaw *et al.*, 2002). Similarly, neutrophils, which are recruited following acute mechanical injury express the ETA receptor subtype and secrete MMP-9, an enzyme capable of cleaving bigET-1 to form bioactive ETs (Fernandez-Patron *et al.*, 2001). The proliferation and phenotypic changes of VSMCs are also likely to be central to explaining the increase in ETB:ETA ratio in intimal lesions, because those of the synthetic phenotype may have increased ETB expression (Eguchi *et al.*, 1994). Finally, it is easy to envisage a system of feedback loops for ET-1 production where many of the cytokines and growth factors, which stimulate ET-1 release are themselves released in response to ET-1 (see section 1.3.1.3).

1.3.3 *Manipulating the ET system in vascular remodelling*

The strongest evidence for the role for the ET system in intimal lesion formation is provided by interventional studies in which the effects of its stimulation or inhibition are investigated using *in vivo* models of disease.

1.3.3.1 *ET-1 administration*

Exogenous ET-1 reliably increases neointima formation in response to balloon injury to the rat carotid artery. Chronic ET-1 administration increases maximum lesion size, collagen content and cell proliferation (Trachtenberg *et al.*, 1993; Barolet *et al.*, 2001) whilst acute administration causes dose-dependent increases in lesion size which are evident 7 days after injury (Douglas & Ohlstein, 1993; Douglas *et al.*, 1994). The target of these treatments has not been determined but, given that a single dose of ET-1 at the time of vascular injury can increase lesion formation, an action on the acute inflammatory response is plausible. A haemodynamic effect must also be considered, but it is difficult to envisage how a transient elevation in blood pressure could have such profound effects on lesion formation.

1.3.3.2 *ECE inhibition*

Combined inhibition of ECE and NEP by chronic administration of phosphoramidon or CGS 26303 reduced lesion size in the rat carotid artery after balloon injury (Minamino *et al.*, 1997; Pham *et al.*, 2002). Selective NEP inhibition, by contrast, did not alter lesion size, suggesting that the effects of these treatments are attributable to their action on ECE (Pham *et al.*, 2002). This is, perhaps, unsurprising given the number of studies demonstrating increased ECE expression in neointimal lesions, but confirms the role of endogenous ET-1 in the response to vascular injury and of ECE in mediating intra-lesion ET-1 synthesis.

1.3.3.3 *Mixed ETA/B blockade*

The number of studies exploring the effects of ET receptor blockade on neointimal lesion formation is far greater than for ECE inhibition (**Table 1-3**). Chronic administration of non-selective ETA/B receptor antagonists (such as bosentan,

enrasentan and TAK 044) reduce neointima size after carotid balloon injury in rats (Douglas *et al.*, 1994; Ohlstein *et al.*, 1994; Douglas *et al.*, 1995a; Tsujino *et al.*, 1995; Chandra *et al.*, 1998) and rabbits (Marano *et al.*, 1998; Azuma *et al.*, 1999). Similarly, ETA/B blockade is reported to reduce lesion size in rabbit carotid artery cuff placement (Marano *et al.*, 1998; Reel *et al.*, 2005) and porcine coronary artery atherectomy models (Sanmartin *et al.*, 2003). Inhibition of neointimal lesion formation by ETA/B blockade has been associated with reduced DNA synthesis (Azuma *et al.*, 1999) but, given that VSMC proliferation can be considered a final common pathway for all processes leading to neointima formation, this does not necessarily indicate a direct effect on these cells.

Not all studies of ETA/B blockade on neointima formation have been positive. In contrast to the acute effects of ET-1 administration, Douglas *et al* (1995a) report no detectable changes in lesion formation following acute treatment with an ETA/B antagonist. This may suggest that, whilst exogenous ET-1 can promote very early, detrimental responses in lesion formation (for example, enhanced neutrophil accumulation), endogenous ET-1 contributes to lesion progression at later stages (for example, increasing macrophage accumulation or VSMC proliferation). Huckle *et al* (2001) also report that even chronic administration of an ETA/B antagonist does not alter lesion size following porcine coronary artery balloon injury, even at a dose 5-fold higher than is necessary to abolish the pressor response to exogenous bigET-1. Whilst this may simply reflect experimental variability or the severity of injury caused, this study is particularly notable for using a model that has been shown to have great predictive value of the clinical efficacy of anti-re-stenotic treatments.

1.3.3.4 *Selective ETA blockade*

ETA antagonist treatment is reported to reduce lesion size in the following models of neointima formation (**Table 1-3**): rat carotid artery after balloon injury (Ferrer *et al.*, 1995), rabbit aorta following diet/balloon injury (Tepe *et al.*, 2002), rat femoral artery after photochemical injury (Takiguchi & Sogabe, 1996), mouse carotid artery following ligation (Murakoshi *et al.*, 2002) and porcine saphenous vein/carotid artery interposition grafting (Wan *et al.*, 2004). Importantly, in contrast to non-selective

Species (Vessel)	Injury	Treatment (Drug)	Lesion Size	Reference
Pig (Carotid)	Balloon	ETA blockade (Atrasentan)	Reduced	(Burke *et al.*, 1997)
Pig (Coronary)	Stent	ETA blockade (Atrasentan)	Reduced	(McKenna *et al.*, 1998)
Pig (Illiac)	Balloon	ETA blockade (Atrasentan)	Reduced	(Burke *et al.*, 1997)
Rat (Carotid)	Balloon	ETA blockade (BMS182874)	Reduced	(Ferrer *et al.*, 1995)
Human (Saphenous)	Vein Culture	ETA blockade (BQ123)	Unchanged	(Porter *et al.*, 1998)
Rabbit (Carotid)	Balloon	ETA blockade (BQ123)	Unchanged	(Azuma *et al.*, 1994)
Rat (Carotid)	Balloon	ETA blockade (BQ123)	Unchanged	(Douglas *et al.*, 1995a)
Pig (Saphenous)	Vein Graft	ETA blockade (BSF302146)	Reduced	(Wan *et al.*, 2004)
Pig (Coronary)	Balloon	ETA blockade (Daruentan)	Reduced	(Dashwood *et al.*, 1999)
Rabbit (Aorta)	Balloon	ETA blockade (Daruentan)	Unchanged	(Tepe *et al.*, 2002)
Rabbit (Aorta)	Balloon/Diet	ETA blockade (Daruentan)	Reduced	(Tepe *et al.*, 2002)
Rat (Femoral)	Chemical	ETA blockade (FR139317)	Reduced	(Takiguchi & Sogabe, 1996)
Mouse (Carotid)	Ligation	ETA blockade (TA0201)	Reduced	(Murakoshi *et al.*, 2002)
Rabbit (Carotid)	Balloon	ETA/B blockade (ATZ1993)	Reduced	(Azuma *et al.*, 1999)
Human (Saphenous)	Vein Culture	ETA/B blockade (Bosentan)	Reduced	(Porter *et al.*, 1998)
Pig (Coronary)	Atherectomy	ETA/B blockade (Bosentan)	Reduced	(Sanmartin *et al.*, 2003)
Rabbit (Carotid)	Balloon	ETA/B blockade (Bosentan)	Reduced	(Marano *et al.*, 1998)
Rabbit (Carotid)	Cuff	ETA/B blockade (Bosentan)	Reduced	(Marano *et al.*, 1998)
Rat (Carotid)	Balloon	ETA/B blockade (Enrasentan)	Reduced	(Chandra *et al.*, 1998)
Pig (Coronary)	Stent	ETA/B blockade (L749329)	Unchanged	(Huckle *et al.*, 2001)
Rat (Carotid)	Balloon	ETA/B blockade (SB209670)	Reduced	(Douglas *et al.*, 1994)
Rat (Carotid)	Balloon	ETA/B blockade (SB209670)	Reduced	(Douglas *et al.*, 1995a)
Rat (Carotid)	Balloon	ETA/B blockade (SB209670)	Reduced	(Ohlstein *et al.*, 1994)
Rabbit (Carotid)	Cuff	ETA/B blockade (TAK044)	Reduced	(Reel *et al.*, 2005)
Rat (Carotid)	Balloon	ETA/B blockade (TAK044)	Reduced	(Tsujino *et al.*, 1995)
Mouse (Carotid)	Ligation	ETB blockade (A192621)	Increased	(Murakoshi *et al.*, 2002)
Human (Saphenous)	Vein Culture	ETB blockade (BQ788)	Reduced	(Porter *et al.*, 1998)
Mouse (Carotid)	Ligation	ETB deletion	Increased	(Murakoshi *et al.*, 2002)

Table 1-3 The effect of chronic endothelin receptor blockade on neointima formation.

Previous studies investigating the ability of chronic ET receptor blockade/deletion to alter neointima formation have demonstrated that both ETA selective and non-selective ETA/B antagonist treatment consistently reduce lesion size in several models. Comparatively few studies have investigated ETB blockade, and results are not consistent.

ETA/B blockade, ETA selective blockade is reported to suppress neointima formation in the clinically-predictive porcine balloon- (McKenna *et al.*, 1998; Dashwood *et al.*, 1999) and stent-induced injury models (Burke *et al.*, 1997). These results indicate that selective blockade of ETA is sufficient to moderate stimulation of lesion development mediated by endogenous ET-1.

The effect of acute ETA blockade on neointima formation has also been investigated. Administration of a single dose of BQ123, a highly selective peptidic ETA receptor antagonist, at the time of balloon injury to the rat carotid artery did not alter lesion development (Douglas *et al.*, 1995a; Douglas *et al.*, 1995b). This is perhaps predictable given that acute ETA/B blockade is similarly ineffective at moderating lesion formation. What is more surprising is that *chronic* BQ123 administration by several means is also reported to be unable to reduce lesion size in the rat and rabbit carotid balloon injury models (Azuma *et al.*, 1994; Douglas *et al.*, 1995a; Douglas *et al.*, 1995b), in which non-peptidic ETA and ETA/B antagonists are effective. As such, BQ123 may have specific qualities that render it unsuitable for the prevention of lesion formation following vascular injury such as poor penetration or accelerated degradation in neointimal lesions. This question is particularly important because Kyriakides *et al* (2003) describe the effect of BQ123 administration on post-PTCA neointima formation in man. No significant reductions in neointimal lesion size were reported with BQ123 treatment as determined by intravascular ultrasound, possibly reflecting the lack of power provided by the small number of patients treated. It is also important to note, however, that BQ123 was administered only acutely, at the time of PTCA and that no animal study of acute ETA/B or ETA selective blockade has ever demonstrated a beneficial effect on neointima formation.

1.3.3.5 *Selective ETB blockade/deletion*

The sheer number of studies demonstrating that both ETA/B and selective ETA antagonist treatment is effective in reducing lesion size following acute vascular injury suggests that any role for ETB is small. This is surprising given the widespread expression of ETB in neointimal lesions, but it is difficult to be sure of this interpretation because no studies (with the following exception) have directly

compared the effects of these treatment modalities. Douglas *et al* (1995a) reported reductions in rat carotid balloon injury-induced lesion formation following treatment with a non-peptidic ETA/B antagonist but not the peptidic ETA antagonist BQ123. This may simply be a reflection of the nature of BQ123, or a differing level of ETA blockade between treatment arms, but may also suggest some role for ETB in exacerbating lesion development in this model. Similarly, intimal hyperplasia in cultured human saphenous veins also involves a detrimental role for ETB because both ETA/B and ETB selective, but not ETA selective antagonists, reduced lesion size in this model (Porter *et al.*, 1998).

Some evidence also points to a protective role for ETB, an action that may be related to several theoretical beneficial effects of ETB receptor stimulation, such as: induction of NO release from ECs and ET-1 clearance. McKenna *et al* (1998), for example, demonstrated substantial reductions in lesion size following coronary artery balloon-injury in pigs treated with a selective ETA antagonist, yet the same group later reported that this model is insensitive to non-selective ETA/B blockade (Huckle *et al.*, 2001). This may imply that ETB blockade can moderate the beneficial effects of ETA blockade on lesion formation. More compelling evidence comes from studies in rescued ETB-deficient mice by Murakoshi *et al* (2002). These animals, made normotensive by restricting dietary salt intake, demonstrated increased lesion size following carotid artery ligation. Additional studies conducted in parallel indicated a similar effect of selective ETB blockade. This was associated with reduced tissue levels of NO oxidation products, suggesting that loss of the EC ETB/ NO pathway may be the mediator of this effect, which may reflect the use of an injury model that does not feature EC denudation. It is difficult to be certain of the role of EC ETB in this setting, however, as neither pharmacological nor conventional knockout tools can distinguish between effects of ETB in EC from those on other cells important to neointima formation. Thus, ETB, possibly that expressed by EC, *may* act to moderate lesion formation following vascular injury and this *may* depend on the mode of injury, with particular emphasis on the state of EC. The scarcity and inconsistent findings of publications addressing this issue, however, prevent firmer conclusions.

1.4 HYPOTHESIS AND AIMS

1.4.1 *Hypothesis*

Acute injury initiates a cascade of thrombotic, inflammatory and mitogenic responses in the vascular wall, leading to intimal thickening by fibro-proliferative lesion formation. Activation of the endogenous ET system by vascular damage contributes to intimal lesion growth by exacerbating inflammatory and proliferative processes. ET-1-mediated stimulation of lesion development appears to be mediated primarily by the ETA receptor subtype but the role of the ETB receptor is less clear. Activation of ETB receptors in vascular smooth muscle and perhaps other cells may stimulate lesion formation whereas stimulation of those expressed by ECs can also mediate effects that might be expected to reduce lesion growth (including stimulation of NO release, endothelial re-growth and ET-1 clearance). Therefore, the work described in this thesis addressed the hypothesis that endogenous ET-1, acting in the ETA receptor, enhances neointima formation following acute vascular injury and that this action is moderated by concurrent stimulation of ETB receptors expressed by the vascular endothelium.

1.4.2 *Aims*

To determine the roles of ET receptor subtypes in the response to vascular injury, the primary aims of this project were:

i) To establish two *in vivo* models of neointimal lesion formation (with and without removal of the endothelium) in the mouse which are amenable to pharmacological and genetic manipulation and to improve existing methodology for the measurement of neointimal lesion morphology.

ii) To determine how selective blockade of ETA or ETB receptors alters neointimal lesion formation and whether concurrent blockade of ETB modulates the response to ETA antagonist treatment.

iii) To determine whether EC-specific deficiency of the ETB receptor gene alters neointimal lesion formation and vascular function.

Chapter 2

Methods

2.1 ANIMALS

All animal experiments carried out in the course of this work were performed in accordance with the Animals (Scientific Procedures) Act (UK), 1986. Mice were bred and housed at the University of Edinburgh Biomedical Research Facility, Little France at 21-22°C and 50% humidity with a 12 hour diurnal light/dark cycle and free access to water and either standard mouse chow (RM1, Special Diets Services, UK) or a drug-containing derivative (section 2.3.2). Unless otherwise stated, experiments were performed on male mice aged 2-4 months. All non-genetically modified mice used were of the inbred C57Bl/6J strain and purchased from Harlan Laboratories, UK.

2.1.1 *Breeding and maintenance of EC-specific ETB knockout mice*

Dr Yuri Kotelevtsev and colleagues have previously bred mice bearing endothelial cell (EC) specific deletion of the ETB receptor in this laboratory (Bagnall *et al.*, 2006). These animals were generated by the cross of floxed ETB mice (FF/--; background: 129/Ola x BKW) with Tie2-Cre transgenic mice (WW/tie2; background: C57Bl/6 x $SJLF_1$), which express Cre recombinase under the control of a promoter region from the angiopoietin-1 receptor, Tie2 (Kisanuki *et al.*, 2001).

Experimental mice were the product of the cross of male FF/tie2 mice with female Cre-naïve FF/-- mice (maintained as an isolated line) to result in progeny of the genotypes FF/-- (Cre-negative control animals) and FF/tie2 (EC ETB-deficient animals). A small minority of the offspring was expected to possess the genotypes F0/-- or F0/tie2, indicating heterozygous deletion of the ETB gene in all cells. This is consistent with observations from several groups that sex-dependent non-specific Cre-mediated recombination of the floxed allele can occur within the gametes even with otherwise specific promoters including Tie2 (Constien *et al.*, 2001; Koni *et al.*, 2001) and AQP2 (Nelson *et al.*, 1998), resulting in deletion of the targeted locus in a non-cell specific manner. To avoid this problem, Tie2-Cre was transmitted exclusively through the male germline which previous experience (Kelland *et al.*, Unpublished data) has shown to dramatically reduce the incidence of non-specific

recombination. Further, because F0/-- mice were detected by routine genotyping, breeding pairs producing animals of this genotype and their offspring were culled without further use. A further consequence of this breeding strategy over many generations is that experimental animals can be considered to possess only the genetic background of the floxed ETB line rather than that of the original Tie2-Cre line.

2.1.2 *Extraction of DNA from tail tips/ ear clips*

Genomic DNA was prepared from tissue samples to allow the genotyping of genetically-modified experimental animals. As mice required ear notching at the time of weaning for identification purposes, the resulting ear clips were collected for DNA extraction to minimize the number of procedures performed on each mouse in accordance with Home Office guidelines. Ear clips, stored at -20°C until needed, were digested in 300µl tail buffer (composition: 200mM Tris HCl pH 8.0, 400mM EDTA, 4.0M NaCl, 10% v/v SDS) containing 10mg.ml^{-1} proteinase K (Roche Applied Biosciences, UK) overnight while continuously rotated at 55°C (Techne HB-1D hybridiser oven). The following day, 10 µl DNAse-free RNAse (40ng.ml^{-1}; Sigma, UK) was added to each sample and incubated (1 hour, 37°C) before cooling on ice until precipitation of SDS was observed. The aqueous phase was carefully removed and the DNA precipitated from this by addition of 300 µl isopropanol and gentle agitation (5 mins), before centrifugation (12000 x g, 2 mins) and removal of the supernatant. Precipitated DNA pellets were washed in 70% v/v ethanol, centrifuged (12000 x g, 2 mins) and the supernatant again discarded. To the resulting washed pellet, 200 µl Tris-EDTA buffer (composition: 100 µM Tris.HCl pH 8, 2 µM EDTA) was added and the samples incubated (30 mins, 37°C) to dissolve DNA before storage at -20°C until needed. Ear clip samples extracted in this way typically yielded final DNA concentrations of the order of 1 µg.ml^{-1}, as determined by spectrophotometry.

2.1.3 *Polymerase chain reaction*

All genetically modified animal strains were genotyped by polymerase chain reaction (PCR) of genomic DNA and separation of products by agarose gel electrophoresis. PCRs for the ETB allele and Tie2-Cre were performed in a total volume of 25 µl containing 0.31 µl each of 200 mM dATP, dCTP, dGTP and dTTP, 0.75 µl of each of the appropriate forward and reverse primer (10 µM), 1 µl of approximately 1 $\mu g.ml^{-1}$ DNA, 2.5 µl of 10X PCR buffer (Bioline, UK), 1.125 µl of 50 mM $MgCl_2$ and 0.2 units of Taq polymerase (Bioline, UK). All reactions were performed in a Peltier Thermal Cycler PT-200 (MJ Research, USA), with heated lid, according to the cycle conditions detailed in **Table 2-1**.

PCR for the ETB allele was performed routinely using the forward primer 5'-TCA GTT GTA ATG AGA CAC AGA C-3' and reverse primer 5'-AGC CAT AAA GTC ACA GCC ATT C-3'. These oligonucleotides flanked a region of up to 2.2kb in length containing both loxP sites, exons 3 and 4 of the ETB allele and an 1166bp deletion in the intron 1 resulting in three possible PCR products: a 2.2kb amplicon 'W' indicating the presence of the wild-type ETB allele, a 1.1kb amplicon 'F' indicating the presence of a loxP-flanked but otherwise intact (non-recombined) ETB allele, and a 186bp amplicon '0' indicating the presence of a recombined loxP-flanked ETB allele (**Figure 2-1**). PCR for the Tie2-Cre transgene was performed using the forward primer 5'-CGC ATA ACC AGT GAA ACA GCA TTG C-3' and reverse primer 5'-CCC TGT GCT CAG ACA GAA ATG AGA-3' as described by (Kisanuki *et al.*, 2001). The presence of Tie2-Cre was indicated by the appearance of a single ~550bp band.

2.1.4 *Agarose gel electrophoresis*

PCR amplification products were separated according to size by agarose gel electrophoresis. Gels were prepared by dissolving agarose (Bioline, UK) to 1% w/v in Tris-Acetic acid-EDTA (TAE) buffer (composition: 40mM Tris base, 20mM acetic acid, 1mM pH 8 EDTA) at 95°C. After cooling to 60°C, ethidium bromide ($0.5\mu g.ml^{-1}$; Sigma, UK) or SYBRsafe (1X; Invitrogen, UK) nucleic acid stains were added and gels allowed to set fully before immersion in TAE buffer. To each 25 µl

Step	ETB allele PCR	Tie2-Cre PCR
1	92°C, 2 mins	92°C, 3 mins
2	92°C, 30 secs	92°C, 30 secs
3	60°C, 30 secs	55°C, 30 secs
4	68°C, 2 mins	68°C, 2 mins
5	Return to step 2, 30 times	Return to step 2, 29 times
6	68°C, 10 mins	68°C, 10 mins

Table 2-1 PCR cycle conditions for genotyping of EC ETB-deficient mice.

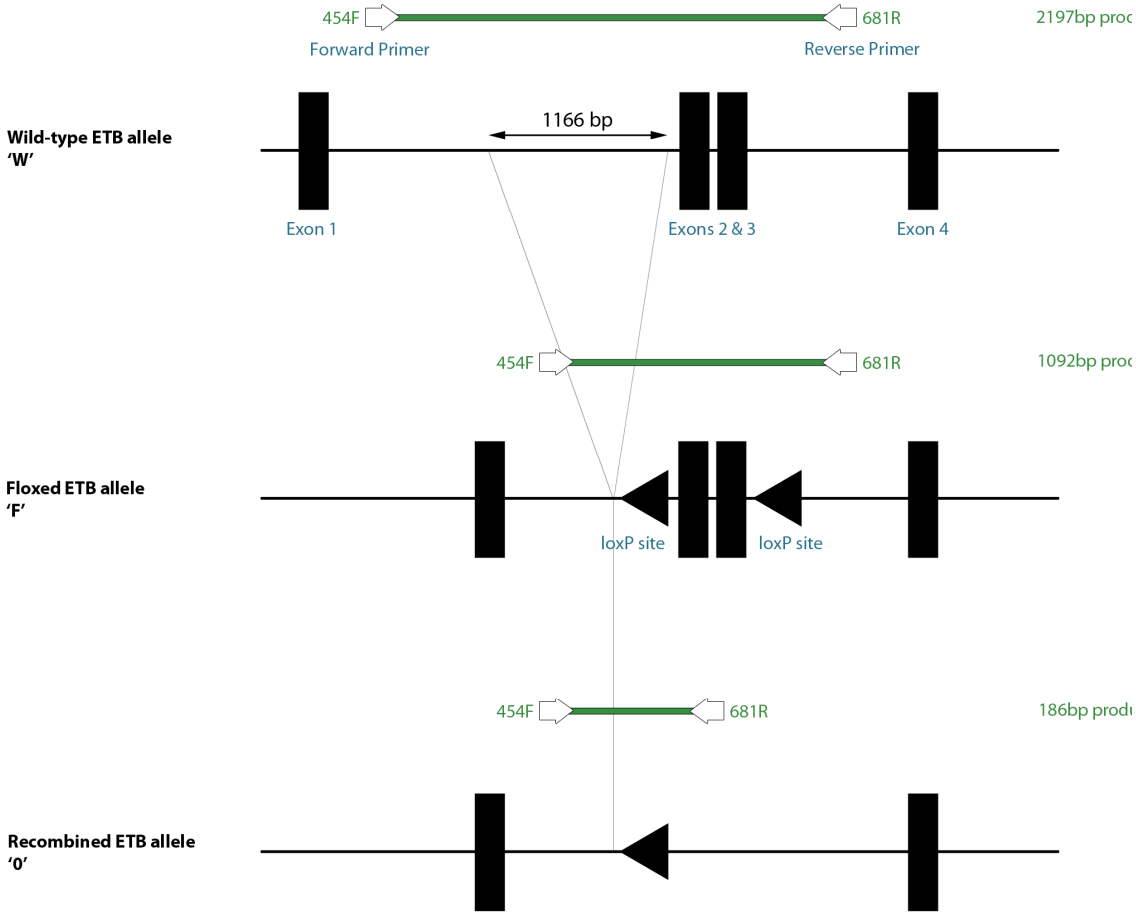


Figure 2-1 Floxed ETB allele and primer schematic.

The ETB allele of floxed ETB mice contains loxP sites flanking exons 2 and 3. The insertion of these required deletion of a 1166bp span of intron 1. The forward primer for ETB allele genotyping lies before this deletion, in intron 1, and the reverse primer in intron 3. Wild-type ETB can be distinguished from floxed ETB by a shortened amplicon which lacks the deleted region of intron 1. The recombined ETB allele can be distinguished by a further abbreviated amplicon which also lacks exons 2 and 3.

PCR product, 2 µl 6x loading dye solution (Promega, UK) was added before loading of samples and separation at 100V for ~1 hour or as needed. To provide reference to molecular mass, standard 100bp DNA ladders (Promega, UK; band sizes: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100bp) were loaded in parallel to PCR product. DNA was visualized by UV transillumination.

2.2 MYOGRAPHY

2.2.1 *Tissue preparation for myography*

For myography studies, mice were killed by CO_2 asphyxiation (Chapter 3) or cardiac puncture/ exsanguination following sodium pentobarbital anaesthesia (Chapter 5; ~120mg/kg i.p.; Ceva Animal Health Ltd, UK). Femoral arteries, from the level of the abdominal wall to the bifurcation of the common femoral artery, were isolated using fine spring scissors and watchmaker forceps (Fine Science Tools, UK) under 10X magnification. Aortas were separated from the thoracic wall and removed with the heart to the point of passage through the diaphragm. Once collected, arteries were immediately immersed in a physiological salt solution (PSS; composition: 119.0mM NaCl, 3.7mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 25.0mM $NaHCO_3$, 1.2mM KH_2PO_4, 27.0µM EDTA, 5.5mM D-glucose) at 4˚C for transport to the myography laboratory.

Here, vessels were secured on plates coated with Sylgard 184 elastomer (Dow Corning, UK) with minutien pins (Fine Science Tools, UK), further cleaned of peri-adventitial fat and connective tissue under 10X magnification and divided into rings ~2mm in length rings under 10X magnification. Femoral artery rings were cut immediately proximal to the common femoral artery bifurcation. Thoracic aortas were separated from the heart and rings taken immediately distal to the aortic arch. Arterial rings were mounted in the organ baths of a Mulvany-Halpern myograph (Multi-myograph 610M, Danish Myotech, Denmark) containing PSS for recording isometric tension. Briefly, rings were threaded over a 40µm diameter tungsten wire (Danish Myotech, Denmark) attached to a micrometer-driven support and secured. A second wire was passed parallel to the first, through the lumen of the vascular ring,

and attached to an opposing support connected to an isometric force transducer (Mulvany & Halpern, 1977). Transducer output was passed to a data acquisition computer (Apple Inc, USA) running Chart 3.4.3 software (AD Instruments, UK) via an analogue/digital converter (Maclab/8, AD Instruments, UK). Exact ring lengths were determined using a calibrated graticule eyepiece (Carl Zeiss Inc, UK). Baths were gradually warmed to 37^{o}C by an integrated heating block and bubbled with 95% O_2 / 5% CO_2 to provide buffering. Over ~30 mins, tensions of 7.5 mN and 8.0mN was gradually applied to aortic and femoral artery rings, respectively, by adjustment of the micrometer. This optimal resting force for each vessel type has been previously determined in our laboratory by normalisation and is in accordance with published literature (Widmer *et al.*, 2006; Bhattacharya *et al.*, 2008).

2.2.2 *Common standard wire myography protocol elements*

To determine the receptor-independent contractility of tissues and to rejuvenate responsiveness following dissection and mounting, bathing media were replaced with iso-osmolar, high K^+ PSS (KPSS; composition: 124.6mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 25.0mM $NaHCO_3$, 1.2mM KH_2PO_4, 27.0µM EDTA, 5.5mM D-glucose). Once the resulting constriction stabilised, tissues were washed with PSS until tension had returned to baseline and the process was repeated twice more. On the third instance, the tension developed in KPSS was recorded as a reference for inherent contractility of that tissue.

For all vascular rings, cumulative concentration-response curves to the α_1-adrenoceptor agonist, phenylephrine (Sigma, UK), were recorded over the range 10^{-9} to 10^{-4}M, using half-log unit steps. At the end of this procedure, rings were washed as necessary with PSS to return tension to baseline, before pre-contraction with a sufficient concentration of phenylephrine to result in 80% of maximal contraction (EC_{80}). Once tension had stabilized, acetylcholine (Sigma, UK) was added in half-log unit concentration steps over the range 10^{-9} to 10^{-4}M to assess endothelium-dependent vasodilatation, before washing with PSS. Subsequent responses to iso-osmolar PSS containing increased K^+ concentration (5-125mM), sodium nitroprusside (10^{-9} to 10^{-5}M; Sigma, UK; in pre-contracted rings), endothelin-1 (10^{-11}

to 10^{-7}M; Merck Biosciences, UK) and/or sarafoxin 6c (10^{-6} or 10^{-7}M; Merck Biosciences, UK; single dose in pre-contracted or un-contracted rings) were obtained as required by the experimental protocol (see appropriate chapters). Where necessary, prior to the exposure to the compound under investigation, inhibitors of nitric oxide synthase (L-NAME; N-nitro-L-arginine methyl ester; $2x10^{-4}$M; Sigma, UK) or antagonists to ETA (BQ123; Cyclo(D-Asp-Pro-D-Val-Leu-D-Trp); 10^{-6}M; Merck Biosciences, UK) or ETB receptors (A192621; (2R,3R,4S)-2-(4-propoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-(N-[(2,6-diethylphenyl]acetamido)pyrrolidine-3-carboxylate; 10^{-7}M; Abbott Laboratories, USA) receptors were incubated with tissues for 30 mins.

2.2.3 *Myography data analysis*

Myography data were recorded as tension developed following administration of drugs. Functional responses produced in response to vasoconstrictor agents were expressed both as absolute force per length of muscle:

$$Absolute\ response\ [mN.mm^{-1}] = \frac{Tension\ [mN]}{2 \times Length\ [mm]}$$

and/or normalised to the tension elicited by the third exposure to KPSS. Responses to vasodilator agents were expressed as a percentage of the pre-existing tension existing immediately before addition of the substance under study. Summary data for concentration-response relationships (E_{max}, maximum response; -log(EC_{50}), concentration eliciting 50% E_{max}) were calculated using Prism 4.0 software (Graphpad software, USA) by iterative least squares fit to the logistic equation:

$$y = \frac{E_{max}}{1 + 10^{(log_{10}EC_{50} - x) \cdot HillSlope}}$$

2.3 TECHNIQUES FOR IN VIVO STUDIES

2.3.1 *Surgical procedures - femoral artery injury*

Vascular injury was inflicted to the femoral artery to enable the study of subsequent neointimal lesion formation. This was achieved by application of the widely used intra-luminal wire-injury model, described by Sata *et al* (2000) and derivatives of the carotid artery ligation-injury model (Kumar & Lindner, 1997), applied to the femoral artery.

2.3.1.1 *Animal preparation, femoral artery cut-down and isolation*

Mice undergoing femoral artery injury surgery were first weighed and placed in an exposure chamber containing 4-5% isoflurane (Merial Animal Health Ltd, UK) to induce anaesthesia. Upon loss of consciousness, animals were transferred to a heated pad, to maintain body temperature, where administration of anaesthetic (~2% isoflurane in oxygen) was maintained via a nose cone. To provide post-operative analgesia mice were administered 0.1 $mg.kg^{-1}$ buprenorphine (Alstoe Animal Health Ltd, UK) by subcutaneous injection. Once surgical anaesthesia was attained, as assessed by pedal withdrawal reflex, mice were rotated to a supine position. Both hind limbs were secured with adhesive tape and the medial surface shaved. On the limb of interest, the inguinal ligament and femoral artery were visualized through the skin and at their convergence, a 5mm transverse incision made. The subcutaneous space was opened and the femoral artery/vein/nerve bundle isolated from the surrounding tissue by blunt dissection with micro-surgery forceps. About the femoropopliteal bifurcation, the femoral nerve was carefully separated from the attached vasculature.

2.3.1.2 *Wire injury*

Approximately 3mm proximal and distal to the arterial branch point, the artery and vein together were looped with 6-0 silk (Fine Science Tools, UK) to which gentle tension was applied with hemostats to temporarily halt blood flow. The popliteal branch was further separated from the adjacent vein and ligated with 6-0 silk. Tension was applied to this ligature with a third hemostat to bring the popliteal artery

from its descending anatomical path into the plane of the femoral artery before peri-adventitial connective tissue was carefully stripped from both arteries. To prevent desiccation, to dilate the femoral artery and to reduce vasospasm following injury, the surgical field was irrigated with 1% w/v lignocaine (Hamlen Pharmaceuticals, UK), as necessary. A transverse arteriotomy was made in the isolated popliteal branch, ~2mm distal to the femoropopliteal bifurcation, through which, a 0.014" diameter straight sprung angioplasty guide wire (Cook Inc, USA) was introduced. The wire was slowly advanced through the femoral artery towards the iliac artery until resistance was met (~1.5 cm) and allowed to distend it for 30 secs. On withdrawal, the proximal loop was rapidly re-tensioned and a further permanent ligature applied immediately proximal to the arteriotomy to prevent egress of blood. Temporary ligatures were removed and reperfusion of the entire femoral artery was observed.

2.3.1.3 *Complete ligation injury*

Proximal to the femoropopliteal bifurcation, the femoral artery and vein were together looped with 6-0 silk (Fine Science Tools, UK) and gentle tension applied with a hemostat to aid access to the branch point. At the point of bifurcation, the artery was carefully separated from the underlying vein and ligated with 6-0 silk. The proximal loop was removed and the reperfusion of the femoral artery and its proximal (but not distal) branches confirmed.

2.3.1.4 *Partial ligation injury*

Partial ligation was achieved as in 2.3.1.3, with the exception that, a permanent ligature was applied to the femoral artery immediately distal to its junction with the popliteal artery, rather than proximal to this bifurcation.

2.3.1.5 *Wound closure and recovery*

Following closure of the external wound with 5-0 silk (Mersilk, Ethicon, UK), in some studies, vascular injury was inflicted on the contra-lateral femoral artery, according to the required technique. Where necessary, animals were ear notched for

identification purposes, and briefly transferred to an empty cage to recover from anesthesia.

2.3.2 *Administration of drugs for in vivo studies*

Pharmacological antagonists were employed to achieve non-cell specific blockade of ETB and ETA receptors in adult animals, thereby avoiding the developmental defects resulting from non-specific genetic deficiency of either receptor. Many potent, selective and well-characterised ET receptor antagonists are available, including the ETA antagonist atrasentan (ABT-627; 2000-fold ETA/ETB selective) and the ETB antagonist A192621 (1300-fold ETB/ETA selective) used in these studies, kind gifts of Abbott Laboratories (Opgenorth *et al.*, 1996; von Geldern *et al.*, 1999). Both drugs are orally-active and were administered in food to achieve respective doses of $10mg.kg^{-1}.day^{-1}$ and $30mg.kg^{-1}.day^{-1}$ assuming consumption of $10g.day^{-1}$ of prepared diet, values suggested by previous studies in our laboratory and in accordance with published values (Opgenorth *et al.*, 1996; Wessale *et al.*, 2002). Drugged diet was bound in gelatin and presented in tubes to prevent dispersion of food around the cage and, therefore, both reduce waste and allow a more accurate monitoring of consumption.

To prepare gelled drug diet, powered beef gelatin (Supercook, UK) was first dissolved in distilled water at $100^{o}C$ and cooled to $40^{o}C$. Drug vehicle was prepared by dispersing methylcellulose (Sigma, UK) in distilled water at $80^{o}C$ to 0.2% w/v before rapid cooling until solvated. Where appropriate, atrasentan and/or A192621 were powdered in a pestle and mortar and dispersed in vehicle solution to concentrations of $83.3\ \mu g.ml^{-1}$ and $250\ \mu g.ml^{-1}$ respectively. Gelatin and drug/vehicle solutions were combined with RM1 powdered mouse chow (Special Diet Services, UK) in the weight ratio 17:15:18. Therefore, each 1kg of food contained 360g powdered diet, 38g beef gelatin dissolved in 302ml distilled water and 300ml 0.2% w/v methylcellulose solution containing, where appropriate, 25mg atrasentan and/or 75mg A192621 in suspension. After vigorous mixing, prepared gel diet was packed in 60ml centrifuge tubes and stored at $-20^{o}C$ until needed.

2.3.3 *Tail plethysmography*

During chronic *in vivo* pharmacological studies, blood pressure measurements were made to verify effective absorption and activity of administered vasoactive drugs. Systolic blood pressure was determined using the technique of tail cuff photo-plethysmography, in which, blood flow through a rodent's tail is monitored by light transmittance (inversely proportional to tail volume) at a point distal to a progressively inflating/ deflating pressurised cuff. The cuff inflation pressure at which cyclical changes in tail volume and, therefore, blood flow are quenched indicates systolic blood pressure.

Rodents dissipate excess body heat, in part, by increasing tail blood flow. To ensure sufficient tail blood flow for photoplethysmography, mice were placed in a 40^{o}C chamber for 10 mins immediately prior to the procedure. Subjects were transferred to a Perspex tubular restraint mounted above a heated pad and an integrated pressure cuff/photosensor sleeve (Harvard Apparatus, UK) was placed around the base of the tail. Animal restraint force was adjusted to maximize comfort and until a regular cyclical tail volume (i.e. pulse) trace was observed on the connected data acquisition computer (International Business Machines Corporation, UK). Pulse traces were recorded while, under computer control, cuff pressures were progressively increased to above, then decreased to below, the pressure at which distal pulse was no longer apparent. For each animal, seven inflate/deflate pressure cycles were performed and systolic blood pressure recorded as the mean measurement from the last four deflate cycles only. Once adequate pressure measurements were acquired, mice were weighed and returned to their cages.

2.3.4 *Perfusion fixation*

Tissue fixation serves to inactivate tissue-catabolising enzymes, prevent growth of microorganisms and provide mechanical strength to delicate structures to allow storage and analysis of biological samples. Aldehyde fixatives such as formalin and glutaraldehyde achieve this by the formation of methylene crosslinks between tissue amine groups. Perfusion of whole animals via the vasculature offers a way to rapidly and evenly deliver fixative to all organs simultaneously, thereby affording rapid

fixation, deep within tissues. Of greater relevance here, perfusion fixation confers sufficient rigidity to blood vessels to maintain their luminal dimensions once excised from their supporting structure.

Trans-cardiac perfusion fixation was performed in mice terminally anaesthetised with sodium pentobarbital (80mg.kg^{-1}, Ceva Animal Health Ltd, UK) administered by intraperitoneal injection. 2 hours prior to the induction of anaesthesia, 2.5mg of 5-bromo-2-deoxyuridine (Sigma, UK), dissolved in 0.5ml 0.9% w/v NaCl, was administered by the same route to label proliferating cells. Following the onset of deep surgical anesthesia, as determined by loss of pedal withdrawal reflex and reduced respiratory rate, a bilateral thoracotomy and transverse sternotomy were made. For the delivery of perfusate, a 23 gauge needle was advanced ~4mm through the free left ventricular wall and secured. A further incision was made in the right atrial wall to allow perfusate to escape. 24ml phosphate-buffered saline (PBS; Sigma, UK) containing 10U.ml^{-1} heparin (Leo Laboratories, UK), was delivered to left ventricle at a constant rate of 6ml.min^{-1} by peristaltic pump (Gilson, UK). After perfusion with heparinised PBS, 24ml 10% neutral buffered formalin (Sigma, UK) was delivered in the same manner to provide fixation and maximize the preservation of tissue morphology. Adequate perfusion was indicated by blanching of the liver and other organs, and proper fixation by characteristic muscular contractions and subsequent rigidity. Following perfusion, blood vessels and organs of interest were dissected and immersed in fixative for a further 48 hours, before transfer to 70% v/v ethanol (Fisher Scientific, UK) for storage until needed.

2.4 HISTOLOGICAL TECHNIQUES

2.4.1 *Tissue processing, paraffin embedding and microtomy*

To enable the cutting of high definition tissue sections for subsequent analysis of morphology and composition, femoral arteries were infiltrated with paraffin wax before sectioning and analysis. Formalin fixed tissues were loaded into identifying cassettes and processed to wax using a Tissue-Tek Vacuum Infiltraton Processor (Sakura Finetek Europe, The Netherlands). Briefly, cassettes were dehydrated in graded concentrations of ethanol (50%, 70%, 95%, 100%, 100% v/v, each for 2

hours; Fisher Scientific, UK), cleared in xylene (two changes, each for 2 hours; Fisher Scientific) and immersed in molten paraffin wax at 60°C (three changes, each for 2 hours; Thermo-Shandon, UK). Each step was performed under vacuum and with agitation to accelerate infiltration of solvents/wax. After processing, femoral arteries were positioned in molten paraffin wax-filled embedding moulds and rapidly cooled on a Tissue-Tek Cryo-console (Sakura Finetek Europe, The Netherlands) to form solid blocks bound to an identifying plastic support. To reduce the quantity of sections to be cut, in some studies, up to 5 arteries were embedded in each paraffin block. This was achieved either by embedding the vessels in 2% w/v agarose (Bioline, UK) prior to processing, or by careful placement and embedding of individually processed vessels in a single embedding mold. In both cases, vessels were arranged in a pattern that allowed the identification of individual arteries within each block, regardless of orientation.

Paraffin blocks were cut into sequential 3µm thick sections using a Leitz 1512 microtome (Leica Microsystems, Germany) and MB35 disposable blades (Thermo-Shandon, UK). Immediately after cutting, ribbons of sections were floated on the surface of a 45^{o}C water bath (Sakura Finetek Europe, The Netherlands), to remove wrinkles, and mounted on Superfrost Plus electrostatically-coated microscope slides (VWR International, UK). Slides were dried overnight at 37^{o}C before further use, to ensure adherence of the sections. Wire-injured femoral artery blocks were sectioned in a repeating pattern, wherein, 16 sections were retained and mounted on 8 slides (2 per slide) followed by discard of the proceeding 100µm of block. Sectioning was continued in this manner until either no vessel was visible, or the remaining vessel was morphologically normal upon microscopic scrutiny, resulting in ~250-300 sections per block. Due to the more focal nature of lesions resulting from femoral artery ligation, when cutting these vessels 10 sections were retained (5 slides, 2 per slide) before discard of only the proceeding 50µm of block, typically requiring only ~80-120 sections to traverse the visibly-injured portion.

2.4.2 *United States trichrome staining*

For routine examination and morphometric analysis, tissue sections were stained using the "United States trichrome" (UST) method (Hadoke *et al.*, 1995) either manually or using an automated cytology stainer (Varistain Gemini, Thermo-Shandon, UK). This technique is a simple combination of a selective elastin stain (Gomori's aldehyde fuchsin) with Gomori's one-step trichrome and thus allows differentiation of collagen (blue-green), elastin (deep purple) and cells (cytoplasm: red; nuclei: black) in a single section.

Gomori's aldehyde fuschin was prepared by dissolving pararosaniline (Sigma, UK) to 1% w/v in 60% v/v ethanol and, to this, addition of 1% v/v HCl (VWR International, UK) and 2% v/v paraldehyde (Sigma, UK). The solution was allowed to 'ripen' for at least two days in sunlight to allow reaction of pararosaniline and acetaldehyde, and vacuum filtered before use through Whatman #1 paper (GE Healthcare, UK). Gomori's trichrome was prepared in distilled water by addition of 0.6% w/v phosphotungstic acid (Sigma, UK), 0.6% w/v chromotrope 2R (Sigma, UK), 0.3% w/v fast green FCF (Sigma, UK) and, after vigorous mixing, 1% v/v acetic acid (Fisher Scientific, UK). Weigert's hematoxylin was prepared from Weigert's solution A and B (Bios Europe, UK) immediately before each use. Acidified potassium permangantate solution (0.3% v/v concentrated H_2SO_4, 0.3% w/v $KMnO_4$) was also freshly prepared before each staining batch, from 3% v/v H_2SO_4 (Fisher Scientific, UK) and 1% w/v $KMnO_4$ (Sigma, UK) stock solutions.

After de-waxing in xylene (Fisher Scientific, UK) and rehydration through graded alcohols (Fisher Scientific, UK) tissue sections were bleached of any inherent pigment by immersion in acidified potassium permanganate and rinsing in oxalic acid (Sigma, UK). Bleached sections were placed in the elastin-stain, Gomori's aldehyde fuschin and differentiated in 70% v/v alcohol. Nuclei were counter-stained with Weigert's acid-fast iron hematoxylin. After rinsing in distilled water, slides were placed in 5% w/v phosphotungstic acid (Sigma, UK) to displace previously applied dyes from collagen fibres and again rinsed in distilled water. In the final staining step, slides were immersed in Gomori's trichrome reagent to stain cytoplasm

and collagen and then rinsed in acetic acid, dehydrated through graded alcohols, and cleared in xylene. Coverslips were mounted with resinous medium (Di-n-butyl phthalate in xylene, Fisher Scientific, UK, or Consulmount, Thermo-Shandon, UK) either manually or with an automated coverslipping device (Consul, Thermo-Shandon, UK). The complete staining protocol is detailed in **Table 2-2**.

2.4.3 *Picro-sirius red staining*

Staining with picro-sirius red, a sensitive and specific collagen fibre stain (Puchtler *et al.*, 1973) was performed to evaluate lesion collagen content. After de-waxing in xylene (2 x 5 mins), re-hydration through graded alcohols (100%, 90%, 70%, each for 2 mins) and rinsing in running tap water (5 mins), slides were immersed in picro-sirius red staining solution. Staining solution was prepared by dissolving the collagen stain Direct Red 80 (Sigma, UK) and plasma counter-stain Fast Green FCF (Sigma, UK) in saturated aqueous picric acid (Sigma, UK), each to 0.1% w/v. After 2 hours, slides were briefly rinsed in tap water, dehydrated in graded alcohols (70%, 90%, 100%, 2 mins each) to xylene (2 x 2 mins) and cover-slipped using resinous media (Consolmount, Thermo-Shandon, UK). Photomicrographs (see section 2.4.4) of picro-sirius red stained sections were quantified by colour deconvolution as for immunohistochemistry (see section 2.4.6.2).

2.4.4 *Digital image acquisition*

To allow analysis of lesion size and composition, colour photomicrographs of histologically- or immunohistochemically-stained tissues were required. These were captured to a data acquisition computer (Dell Inc, UK) running MCID Basic 7.0 software (Imaging Research, USA) under 10X objective magnification (Axioskop stage microscope, Carl Zeiss Inc, UK) by a monochromatic CCD camera (CoolSNAP Colour, Photometrics, USA) with liquid crystal RGB filter (MicroColour, CRI Inc, USA).

2.4.5 *Morphometry*

Morphometric analysis was performed from photomicrographs of femoral artery sections following UST staining to aid in identification of the elastic laminae. From

Step	Reagent	Duration
1	Xylene (VWR International, UK)	2 x 5 mins
2	100% v/v ethanol (Fisher Scientific, UK)	2 x 5 mins
3	95% v/v ethanol (Fisher Scientific, UK)	1 x 5 mins
4	Running tap water	1 x 5 mins
5	0.3% w/v $KMnO_4$ (Sigma, UK) in 0.3% v/v H_2SO_4 (Fisher Scientific, UK)	1 x 1 min
6	Running tap water	1 x 1 mins
7	2% v/v oxalic acid (Sigma, UK)	1 x 5 secs
8	70% v/v ethanol (Fisher Scientific, UK)	1 x 5 mins
9	Gomori's aldehyde fuchsin	1 x 5 mins
10	70% v/v ethanol (Fisher Scientific, UK)	1 x 2 mins
11	Running tap water	1 x 5 mins
12	Weigert's hematoxylin (Bios Europe, UK)	1 x 5 mins
13	5% w/v phosphotungstic acid (Sigma, UK)	1 x 5 mins
14	Running tap water	1 x 2 mins
15	Gomori's trichrome	1 x 20 mins
16	0.2% v/v acetic acid (Fisher Scientific, UK)	1 x 1 min
17	95% v/v ethanol (Fisher Scientific, UK)	1 x 1 min
18	100% v/v ethanol (Fisher Scientific, UK)	2 x 2 mins
19	Xylene (VWR International, UK)	2 x 2 mins

Table 2-2 United States Trichrome staining protocol.

these, measurements of medial area, intimal area and luminal area were made. Media was defined as the region delineated by the internal (IEL) and external (EEL) elastic laminae; intima by the region within the IEL composed of cellular or stable thrombotic material; and lumen by any remaining "empty" area within the IEL. Borders of the relevant areas were traced using Photoshop CS3 Extended software (Adobe Systems Inc, USA) and each area calculated according the scale: 1 pixel = 0.5126 μm^2, as determined by imaging of a graticule slide. From these values, additional morphometric parameters of intima/media ratio and stenotic ratio were calculated according to the following equations:

$$Intima/Media\ Ratio = \frac{Intimal\ Area}{Medial\ Area}$$

$$Stenotic\ Ratio = \frac{Intimal\ Area}{Intimal\ Area + Lumenal\ Area}$$

2.4.6 *Immunohistochemistry*

Immunohistochemistry is a powerful technique for conferring molecular-specificity to histological preparations first described by Albert Coons in 1941. Common implementations of the original process involve visualization of the binding of a specific antibody/anti-serum to its target, by fluorescence or the enzymatic development of a chromogenic substrate, and necessitate several interleaved amplification steps depending on the abundance of antigen and quality of antibody. In this work, immunohistochemistry was performed according to the indirect labeled streptavidin-biotin (LSAB) method and reported by the peroxidase-catalysed oxidation of 3,3'-diaminobenzidine to an insoluble phenazine polymer, as detailed in **Figure 2-2**. Because chemical fixation, especially by aldehydes, can disturb the structure and accessibility of antigens of interest, protease-induced epitope retrieval (PIER) was used, where necessary, to facilitate specific binding of the primary antibody. To increase throughput, immunohistochemistry reactions were carried out using the 'semi-automated' Sequenza system (Thermo-Shandon, UK). This comprises an electrostatically-charged coverplate, which is affixed to each slide to

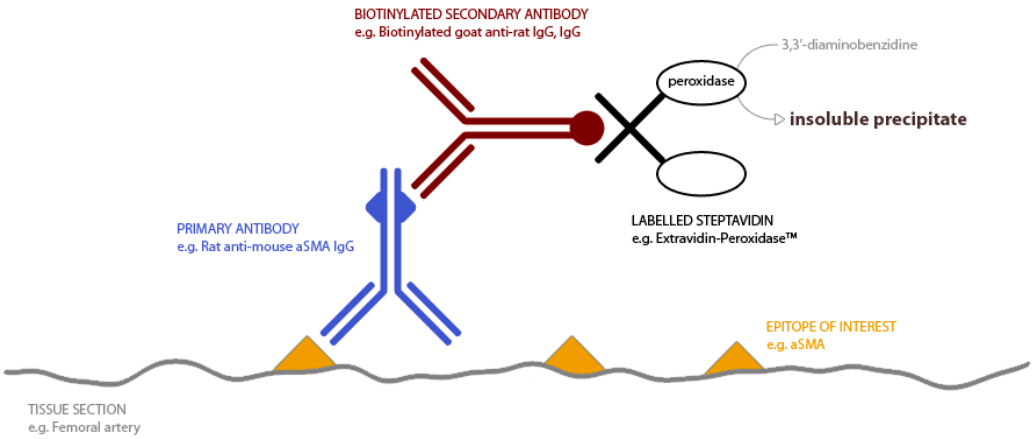


Figure 2-2 Schema for immunohistochemistry by the indirect labelled streptavidin biotin method.

Primary antibodies are allowed to bind to epitopes of interest on tissue sections. To these bind secondary antibodies which are reactive to immunoglobulins of the species and isotype of the primary antibody. Secondary antibodies employed are pre-labelled with biotin. After formation of the epitope-primary-secondary antibody complex, sections are treated with a conjugate of streptavidin and horseradish peroxidase. Peroxidase catalyses conversion of the 3,3-diaminobenzidine to an insoluble brown product. Thus, after incubation with this substrate, the location of the original epitope of interest can be visualised. The use of these successive steps provides simple amplification as the stoichiometry of each reaction is greater than 1:1. αSMA, α-smooth muscle actin; IgG, immunoglobulin G.

form a narrow gap over the slide surface and is fed by a small reservoir to which reagents can be added. The charged surface of the coverplate ensures free and rapid passage of solutions, facilitating washing of slides, while capillary action dictates that the final ~80µl of added liquid is retained, allowing incubation of reagents with tissue sections and protecting slide contents from desiccation.

To provide routine validation of immuno-staining procedures, at least four control slides were included in each experimental batch. Three negative control slides were included to ensure positive staining was not explicable by non-specific binding of a reagents: two reagent negatives in which both the target tissue and a tissue highly expressing the antigen of interest were treated with a control reagent in place of primary antibody (such as a non-reactive antibody of the same immunoglobulin isotype at a matched concentration) and a tissue negative in which the relevant antigen is not expected to occur. To confirm the effectiveness of each immuno-staining batch and provide a references for colour deconvolution (see section 2.4.6.2), a positive control slide bearing a section of tissue expected to strongly express the antigen of interest was also stained in parallel to experimental sections. Details of control tissues/reagents for each immunohistochemistry protocol used are given in **Table 2-3** and examples of staining in **Figure 2-3**.

2.4.6.1 *Immunohistochemical staining protocol*

All immunohistochemistries in this work were carried out according to the following general protocol. Antibody-specific variations are given in **Table 2-4**. Paraffin sections were first baked at 55°C to ensure adherence of tissues to slides. After 30 mins, slides were removed from the oven, de-waxed in xylene (2 changes, each for 5 mins), rehydrated through graded ethanols (100%, 90%, 70%, each for 2 mins) and rinsed under running tap water (5 mins).

For anti-BrdU staining, slides were pre-treated by enzymatic digestion to restore the confirmation of antigens lost to fixation. To achieve this, slides were immersed in 1mg.ml^{-1} porcine pancreatic trypsin (Sigma, UK) dissolved in 1mg.ml^{-1} $CaCl_2$

Antigen	Tissue Positive Control	Reagent Negative Control (reagent; dilution; supplier)	Tissue Negative Control
αSMA	Uninjured femoral artery (media) Kidney (muscular arteries)	NS-1 myeloma ascites fluid, 1/400 Sigma, UK	Kidney (tubules)
Mac2	Spleen 14-day injured femoral artery	$IgG2_{a\,\kappa}$, 1/3000 BD Pharmingen, UK	Uninjured femoral artery
BrdU	Ileum from BrdU treated mouse	NS-1 myeloma ascites fluid, 1/800 Sigma, UK	Ileum from BrdU untreated mouse

Table 2-3 Control tissues and reagents for immunohistochemistry reactions.

αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine.

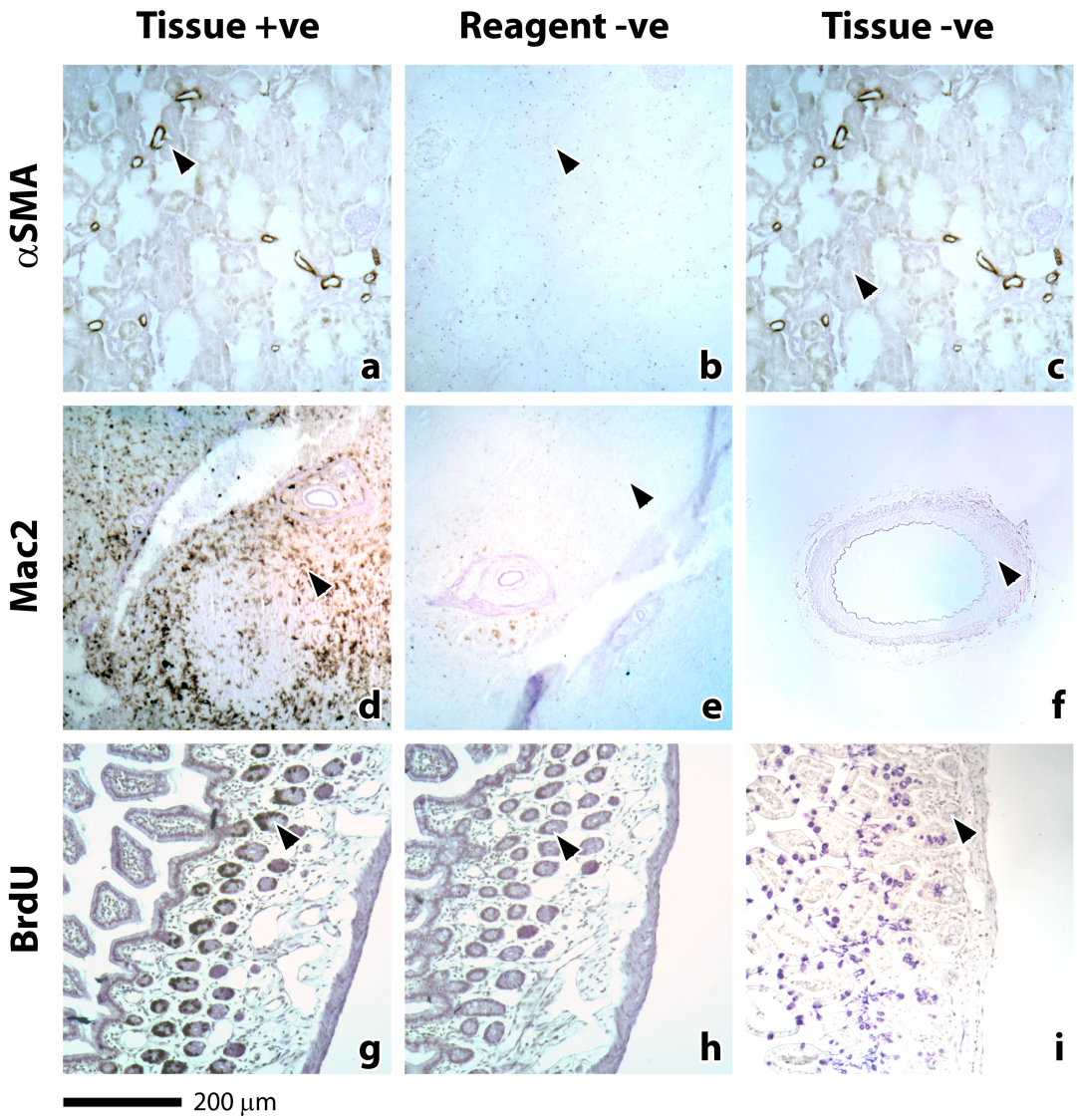


Figure 2-3 Example immunohistochemistry staining in control tissues.

αSMA immunohistochemistry in kidney sections reveals strong staining of vascular smooth muscle in renal blood vessels (a) and no staining in tubular epithelial cells (c). When primary antibody is substituted for ascites fluid from NS-1 myeloma cells, no immunoreactivity is observed in any renal cell type (b). Mac2 staining is abundant throughout the red pulp of the spleen (d) and this is abolished upon substitution of primary anti-Mac2 antibody with an irrelevant antibody of the same species and isotype (e). In uninjured femoral arteries, no Mac2 immunoreactivity is apparent (f). Intense BrdU immunoreactivity is present in the crypts of intestinal villi from mice treated with BrdU (g) but is completely absent in untreated animals (i). BrdU staining is also abolished by substitution of the primary antibody with ascites fluid from NS-1 myeloma cells (h).

Primary Antibody **(antibody; dilution; incubation; supplier)**	**Pre-treatment** **(composition)**	**Blocking** **(composition; incubation)**	**Secondary Antibody** **(antibody; dilution; incubation; supplier)**	**E-P** **(dilution)**
Mouse anti-mouse αSMA Clone 1A4 1/600, 30 mins @ 20°C Sigma, UK	None	1: 2.5% MP, 2.5% BSA 60 mins 2: 20% GS, 30 mins	Goat anti-mouse, 1/400, 30 mins @ 20°C Vector Laboratories, UK	1/400
Rat anti-mouse Mac2 (galectin-3) Clone M3/38 1/6000, overnight @ 4°C Cedarlane Laboratories, USA	None	1: 20% GS, 1% BSA, 30 mins	Goat anti-rat, 1/200, 30 mins @ 20°C Vector Laboratories, UK	1/200
Mouse anti-BrdU Clone BU-33 1/800, 1 hr @ 20°C Sigma, UK	1: Trypsin 2: HCl	None	Goat anti-mouse, 1/200, 30 mins @ 20°C Vector Laboratories, UK	1/200

Table 2-4 Details for specific immunohistochemistry reaction protocols.

E-P, extravidin-peroxidase; PECAM-1, platelet-endothelial cell adhesion molecule-1; GS, normal goat serum; BSA, bovine serum albumin; αSMA, α-smooth muscle actin; MP: milk powder (J Sainsburys Ltd, UK); BrdU, 5-bromo-2-deoxyuridine.

(Sigma, UK) at pH 7.8. Sections were incubated for 30 mins at 37°C before rinsing in PBS (5 mins). Sections were subsequently subject to denaturation (acid hydrolysis) of DNA by incubation of sections with 2N HCl (30 mins at 37°C, Sigma, UK) to allow access of antibody to incorporated BrdU before further rinsing.

For all protocols, slides were installed in Sequenza coverplates trapping 80µl of PBS between slide and coverplate and ensuring the capillary gap was free of air bubbles. Sequenzas were twice flushed with PBS before the addition of 3% v/v H_2O_2 solution (Sigma, UK) to block endogenous peroxidase activity. After 5 mins, Sequenzas were again twice flushed with PBS. To reduce non-specific binding of primary antibodies, sections were incubated with various blocking solutions, most commonly PBS containing 1% w/v bovine serum albumin (BSA, Sigma, UK) and 20% normal serum from the species in which the appropriate secondary antibody was raised. Blocking solutions were allowed to equilibrate with tissues, as required, at room temperature. Without washing, primary antibody diluted in 1% w/v BSA in PBS was added to the Sequenzas and incubated for the required time and at the appropriate temperature (**Table 2-4**). Primary antibody solution was removed from slides with three 5 min washes in PBS and replaced by secondary antibody solution, also diluted in 1% w/v BSA in PBS. After the required incubation at room temperature, slides were again washed with PBS (3 x 5 mins). Extravidin-Peroxidase (Sigma, UK), a conjugate of streptavidin and horseradish peroxidase, was diluted as necessary in PBS containing 1% w/v BSA and incubated with slides for 30 mins before final washing in PBS (3 x 5 mins). To visualise the distribution of HRP, and, therefore, antibody binding to tissues, a proprietary solution of H_2O_2 and 3,3'-diaminobenzidine (DAB+, prepared according to manufacturer's instructions, Dako Cytomation Ltd, UK) was applied to tissue sections. To determine the appropriate incubation time, positive control slides were removed from Sequenzas, tissue sections circled using a hydrophobic barrier pen (Calbiochem, UK) and incubated with DAB+ solution while the development of brown precipitate was observed under 40X magnification. DAB+ was applied to remaining slides within Sequenzas for the determined time (typically 1-2 mins). The reaction was quenched by flushing Sequenzas with tap water, removal of slides, and immersion in running tap water for a further 5 mins. Sections were counterstained by

5 brief dips in Harris's aluminum hematoxylin solution (Fisher Scientific, UK) before bluing in 0.1% v/v NH_3OH solution (Sigma, UK) for 5 mins, dehydration through graded alcohols (70%, 90%, 100%, 2 mins each) to xylene (2 changes, 2 mins each) and coverslip mounting using resinous medium (Shandon Consulmount, Thermo-Shandon). In some studies, immuno-stained slides were dehydrated and coverslipped without counterstain to improve clarity and aid quantification.

2.4.6.2 *Quantification*

Quantification of immunoreactivity was performed from photomicrographs of immuno-stained tissues, acquired as described in section 2.4.4. To increase accuracy and reduce the potential for bias, positive staining was indentified using a semi-automated colour de-convolution process (**Figure 2-4**) with Photoshop CS3 Extended software (Adobe Systems Inc, USA). Briefly, colour values corresponding to DAB+ precipitate were recorded from positive control slides stained in parallel to sections of interest. Colour definitions were applied to images of experimental sections for the calculation of the appropriate quantity. αSMA and Mac2 immunoreactivity were expressed as the percentage of area occupied by DAB+ precipitate within specific areas (neointima, media), delineated as defined in section 2.4.5. For the measurement of BrdU immunoreactivity, the number of distinct BrdU positive (i.e. visibly DAB stained) within these defined regions were counted manually. To avoid problems of inter-batch variability in immunohistochemistry reactions, quantitative comparisons were only made between slides stained in parallel.

2.5 OPTICAL PROJECTION TOMOGRAPHY

Optical projection tomography (OPT) is a recently described 3D imaging technique (Sharpe *et al.*, 2002) with similarities to optical computed tomography (CT) and single photon emission CT (SPECT). Compared with rival methods, OPT is able to image samples too large for (single photon) confocal microscopy and optical coherence tomography systems while providing images of greater resolution than those produced by μMRI and CT systems and at much lower cost. Serial histological

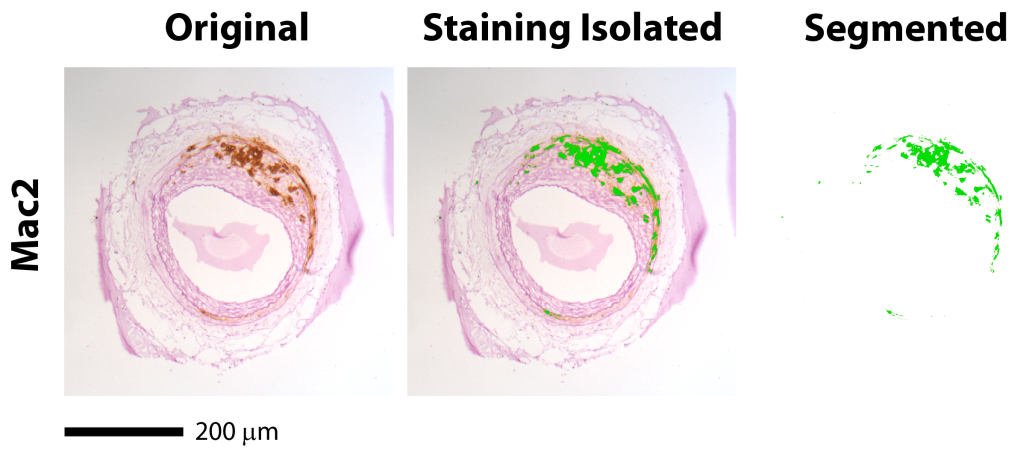


Figure 2-4 Example colour deconvolution of immunohistochemistry staining.

Colour deconvolution allows the area of antigen expression to be calculated from immunohistochemically stained sections. To do this, photomicrographs of immunoperoxidase-stained vessels are recorded (a), and in these colour values corresponding to DAB precipitate are determined from positive control slides (b, highlighted in green). Images are then segmented according to this definition (c) and the area expressed as a fraction of the region of interest (e.g. neointima, media).

sections offer superior resolution and flexibility but, unlike OPT, 3D reconstructions of 2D sections are desctructive, slow and labour-intensive to produce, do not retain the true confirmation of the sections in 3D space and do not possess the accurate z-axis position information necessary for volumetric measurements.

The optical projection tomograph (Bioptonics 3001, Bioptonics, UK) comprises a diffuse light source, illuminating a rotating, biological sample, suspended in a refractive index matching solution. Adjustable optics focus light from a plane of interest in the sample onto a charged coupled device (CCD), which captures intensity data to a connected computer (Dell Inc, UK) for acquisition and reconstruction. The device operates in two modes – transmission OPT (tOPT) and emission OPT (eOPT). In tOPT, a polychromatic visible and/or infrared source is employed and light transmitted through, rather than absorbed or reflected by, the sample, is focused to the CCD, which, as in x-ray or optical CT, captures shadow images. In eOPT, a metal-halide ultraviolet source illuminates the sample through a chromatic filter selective for an excitation wavelength of interest. Photons emitted by excited flourophores within the specimen are focused through an emission filter to the CCD, a process analogous to SPECT. In both modes, a 3D image is reconstructed using a modified computed tomography back-projection algorithm from multiple single projections covering 360º of sample rotation. In these studies only eOPT was employed as the small size and great transparency of refractive index matched femoral arteries precluded the capture of useful opacity data.

2.5.1 *Tissue preparation, sample embedding and mounting*

Femoral arteries intended for OPT imaging were excised from mice following trans-cardiac perfusion fixation under terminal pentobarbital anesthesia (see section 2.3.4). Samples were cleaned of peri-adventitial fat and post-fixed by immersion in 10% neutral buffered formalin (Sigma, UK) for 48 hours. Low melting point agarose (Invitrogen, UK) was dissolved in distilled water to 1.5% w/v at 90°C. Agarose solution was allowed to cool (40-50°C) before filtering through Whatman 113V paper (GE Healthcare, UK) to eliminate particulate contaminants. Each fixed femoral artery was suspended in 40ml of filtered agarose solution and rapidly cooled (<20°C)

to accelerate gelling of the agarose. Gelled agarose blocks were bonded to magnetic OPT mounts with cyanoacrylate adhesive (Henkel, UK) so each contained artery was suspended in the axis of the mount. The mounted blocks were trimmed to a conical shape to minimize back-reflection of light within the plane of scanning. Mounted samples were dehydrated in methanol (Fisher Scientific, UK) for 24 hours and transferred to a refractive index-matching solution (BABB) of 34% v/v benzyl alcohol (Acros Organics, UK), 66% v/v benzyl benzoate (Acros Organics, UK) and allowed to clear for >24 hours.

2.5.2 *Scanning and tomographic reconstruction*

Before each session, the optical projection tomograph was registered to the axis of sample rotation by scanning a calibration pin during its rotation over 360º and calculating an appropriate correction factor. Tissue sample mounts prepared as described (section 2.5.1) were installed in the scanning chamber by magnetic attachment to an internal support. Under bright-field conditions (i.e. illuminated by white light), the placement of each mount was adjusted by the control of integrated pusher devices and variable vertical positioning, until the sample was seen to revolve about its own axis in the centre of the field of view. Optical magnification was set to 1 pixel = 4µm and 1 pixel = 6µm respectively for ligation- and wire-injured femoral arteries, in each case, the maximum magnification possible when imaging the entire lesion-afflicted length of vessel. For scanning, samples were illuminated by a metal-halide UV source with 425/40nm band-pass excitation filter and emission 475nm low-pass emission filter ('GFP filter', a filter set seen to strongly detect auto-fluorescence). CCD exposure time was adjusted to maximise the dynamic range of the resulting image, typically 300-600ms. The scanner was finely focused to a point ¼ of the sample thickness from its front as described by Walls *et al* (2007). Raw data were acquired by automated capture of projection images (1024x1024 pixels) at rotation increments of 0.9º to give 400 images covering 360º of rotation. After scanning, mounted femoral arteries were cleared of BABB by immersion in methanol for >24 hours. Tissues were removed from mounts and trimmed of excess agarose before processing for histology as described in section 2.4.1.

Projection images were manually evaluated for focus and rotation misalignment using supplied software (Data Viewer; Skyscan, Belgium) before computed tomography reconstructions were calculated. Hamming filtered back-projection of the axial plane was performed by standard methods using NRecon software (Skyscan, Belgium). For each dataset, misalignment correction was applied to compensate for minor and unavoidable defects in the apparent axis of rotation. The correction required was estimated by manual scrutiny of test reconstructions of 2D planes within each data set, calculated about a value determined in software by the comparison of the 0° and mirror inverted 180° projections.

2.5.3 *Quantification*

CT data analysis software (CTan; Skyscan, Belgium) was used for the quantification of reconstructed OPT data. For each vessel scan, a vertical region of interest was defined containing the 2.5mm (ligation-injury) or 5mm (wire-injury) immediately proximal to the bifurcation of the common femoral artery. These lengths were sufficient to encompass the entire neointimal lesion in each respective model. Within this, for every 50^{th} scan-line, the border between media and neointima (i.e. the position of the internal elastic lamina) was estimated and traced. This was possible due to the greater fluorescent signal emitted by the media and by observation of the luminal conformation. Intima/media borders for the interleaved scan lines were interpolated in software, and the fit verified and corrected where required. This defined three dimensional volume was further segmented according to a manually-defined intensity threshold to a produce a binary image set in which white pixels represented neointima and black pixels represented patent lumen. Volumes (μm^3) of each segment were calculated to give measures of neointimal and luminal volume, and the fraction of the total defined volume occupied by neointima, a volumetric 'stenotic ratio', was calculated.

2.6 STATISTICS AND DATA ANALYSIS

Statistical testing and data analysis was performed using Prism 4.0 software (Graphpad software, USA). Data sets comprising two groups were compared using

Student's un-paired t-test (Chapters 3 and 5). Where comparison between three or more groups was required, data were tested by one-way ANOVA with Dunnett's post-hoc test (Chapter 4). Myography data were analysed by unpaired t-test of summary stats, and two-way ANOVA of complete concentration-response data sets. In all studies, n refers to the number of individual animals from which data were acquired. Where the same data were obtained from multiple samples from a single animal (e.g. myography responses in multiple artery rings), the mean of these responses was recorded as n=1.

Chapter 3

Functional and morphological consequences of acute femoral artery injury

3.1 INTRODUCTION

An obvious pre-requisite to studies of the role of endothelin (ET) receptors in the response to vascular injury is the ability to induce injury and quantify the response. For previous studies in this laboratory investigating the role of glucocorticoids in neointimal proliferation (Dover *et al.*, 2007), two models of acute vascular injury have been utilised. The first is the well-characterised and widely published model of intra-luminal wire-injury to the mouse femoral artery, originally described by Sata *et al* (2000). The second involves ligation of the popliteal artery, just distal to the femoropopliteal bifurcation – a modified wire-injury sham procedure. Lesions resulting from popliteal artery ligation, however, are extremely focal and far smaller than those induced by carotid artery ligation (Kumar & Lindner, 1997) complicating analysis of lesion size. Studies of ligations applied about the carotid artery bifurcation indicate that the position of the ligation has a dramatic effect on the degree of lesion formation (Kumar & Lindner, 1997; Sullivan & Hoying, 2002; Korshunov & Berk, 2003). The effect of alternative ligations to the femoropopliteal bifurcation on neointima formation in this location is unknown and, as such, it may follow that optimising the site of the ligation may provide a greater stimulus for neointima formation and result in larger, more easily analysed lesions.

The issue of analysing sporadic, focal lesion formation might also be addressed by a utilising a method of visualising complete lesions in three-dimensions. This would provide further advantage in the ability to consider volumetric parameters of lesion formation resulting from any injury: a more robust and sensitive measure than the traditionally used maximum lesion cross-sectional area (McAteer *et al.*, 2004). Few techniques have been successfully applied to 3D analysis of vascular morphology in mouse arteries, and those that have, such as magnetic resonance imaging (MRI), have many practical limitations (cost, imaging time and resolution; McAteer *et al.*, 2004). Optical projection tomography (OPT) is a recently-described 3D imaging technique originally applied to the study of mouse embryos (Sharpe *et al.*, 2002). In principal, OPT has many features that make it attractive for imaging isolated mouse arteries – it offers high, isotropic resolution for specimens of this size, short acquisition times, and is non-destructive to the specimen (Sharpe *et al.*, 2002;

Alanentalo *et al.*, 2007). Whether this technique is suitable for determining the extent of lesion formation in injured mouse arteries in practice has yet to be determined.

These caveats aside, by utilizing ligation and wire-injury models in parallel (potentially in the same animals) additional insight can be drawn. These models are complementary because they differ in several ways including the nature of injury and the origin of neointimal cells (Kumar & Lindner, 1997; Sata *et al.*, 2000; Tanaka *et al.*, 2003). Perhaps the most critical difference between models in the context of these studies is the relative status of endothelial cells (EC) as wire- but not ligation-induced injury effectively denudes the endothelium (Sata *et al.*, 2000). Previous studies in this laboratory have demonstrated the regeneration of EC-like cells by 14 days post-injury in this model (MacDonald *et al*, Unpublished data) but the degree and time-course of functional recovery is not known. Further, in models of both 'endothelium-denuding' intra-luminal injuries (Azuma *et al.*, 1990; Heijenbrok *et al.*, 1998; Miller *et al.*, 2003; Liao *et al.*, 2007), and 'endothelium-intact' peri-vascular injuries (De Meyer *et al.*, 1991; De Meyer *et al.*, 1992), some degree of endothelial dysfunction is commonly reported. Any impairment of endothelial function following femoral artery wire- and ligation-injury is of great importance as a deficit here may complicate interpretation of the hypothesized protective role for ETB receptors in EC in the response to acute vascular injury.

The experiments conducted in this chapter were therefore intended to determine the effects of femoral artery wire- and ligation-injuries on vascular structure and function, using histology, myography and 3D tomographic imaging. As such, the specific aims are:

a) To determine whether alternative ligations of the femoral artery (proximal or distal to the femoropopliteal bifurcation) elicit robust neointimal thickening.
b) To visualise lesion distribution resulting from wire and ligation-injury using OPT and evaluate the potential of this technique for the quantitative analysis of lesion size.
c) To determine how endothelium-dependent vasodilatation and other aspects of vascular function are altered by wire- and ligation-injury.

3.2 METHODS

3.2.1 *Animals*

All studies presented in this chapter were performed using 20-30g C57Bl/6J mice purchased from Harlan, UK. Animals were allowed to acclimate to their environment for at least 1 week prior to their further use. For vascular injury studies, identifying ear notches were cut at the time of surgery.

3.2.2 *Femoral artery injury*

To inflict femoral artery wire-injury, in order to determine any effect on vascular function (**Figure 3-1**), a straight sprung angioplasty guide wire (0.014" diameter) was advanced in a retrograde manner through an arteriotomy in the popliteal artery into the femoral artery, before withdrawal of the wire and ligation of the popliteal artery proximal to the arteriotomy (Sata *et al.*, 2000). The procedure was performed on the left femoral artery only. Animals were killed by asphyxiation in CO_2 14 days post-injury, a time point at which an EC-like monolayer of von Willebrand Factor immunoreactive cells has returned (MacDonald *et al*. Unpublished data) but at which lesion formation is sufficiently moderate to permit mounting of vessels in a myograph (Sata *et al.*, 2000). Left (injured) and right (uninjured) femoral arteries were harvested for parallel functional evaluation.

For investigations into the morphological and functional consequences of femoral artery ligation, two groups of animals were employed (**Figure 3-1**). In the first group, following isolation of the left femoral artery from the surrounding tissue and the adjacent vein and nerve, a 5-0 silk ligature was applied to the femoral artery, just distal to the femoropopliteal bifurcation. This study group is referred to as partial ligation. In the second group, the procedure was identical except that the ligature was applied to the femoral artery across the bifurcation. This study group is referred to as complete ligation. To the right femoral artery, in both groups, a sham operation was performed, in which, after isolation of the artery, a ligature was passed under the appropriate point, but removed rather than tied. Animals were killed 28 days post-injury by CO_2 asphyxiation. This time-point was chosen because it represents

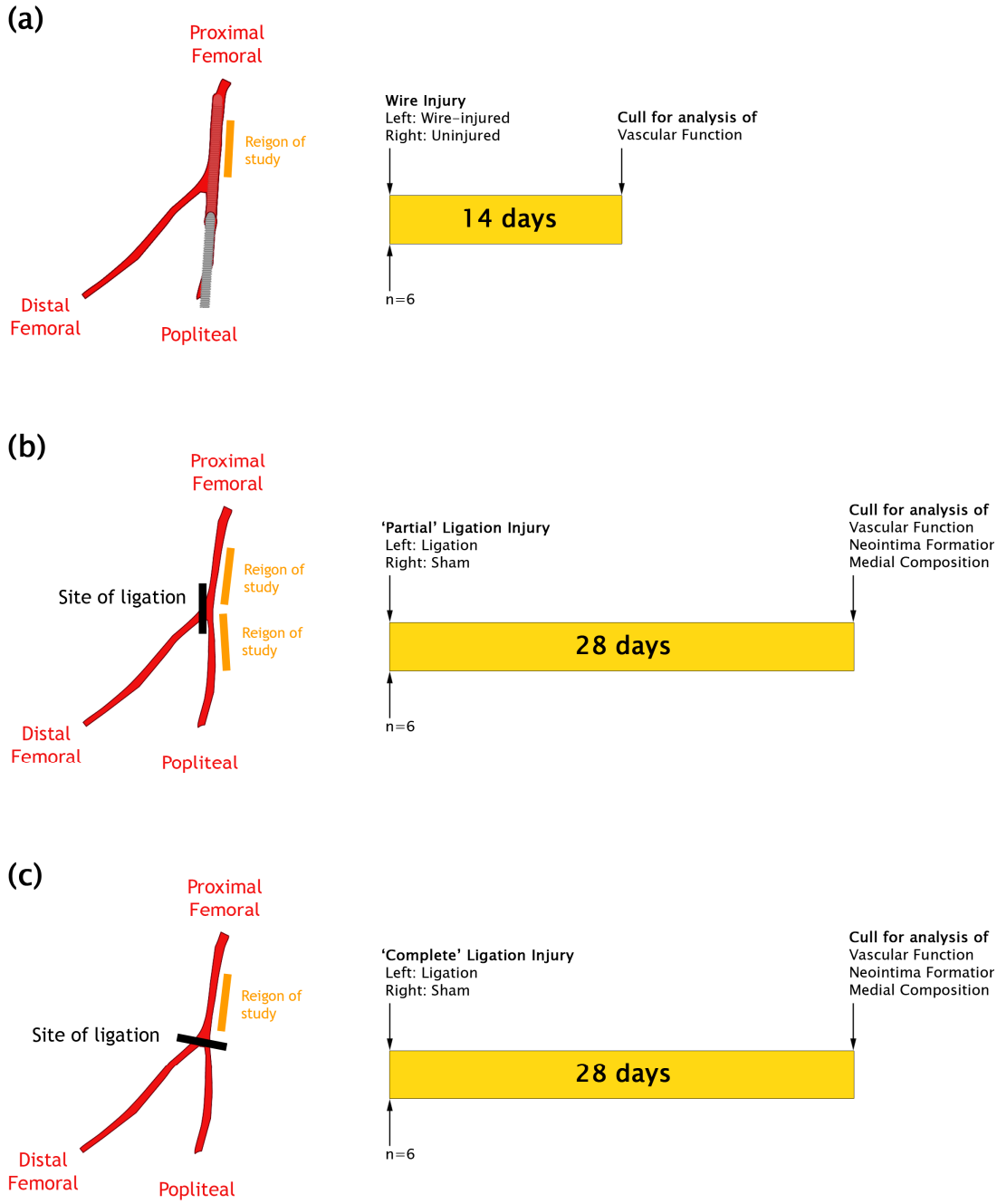


Figure 3-1 Study protocol for investigation of the structural and functional consequences of femoral artery injury.

Wire-injury entails wire insertion into the proximal femoral artery through an arteriotomy in the popliteal artery (a). Vessels were collected after 14 days and subject only to functional investigation in the region just proximal to the femoropopliteal bifurcation. 'Partial' ligation entails ligation of the femoral artery just distal to the bifurcation (b). Vessels were collected after 28 days and subjected to functional and morphometric evaluation. Analysis was performed on sections from the femoral artery proximal to the ligation, and the popliteal artery just distal to it. 'Complete' ligation entails ligation of the femoral artery over its junction with the popliteal artery (c). Vessels were collected after 28 days and subject to functional and morphometric evaluation. Analysis was performed only on the section of femoral artery immediately proximal to the ligation.

maximum stable lesion size in the similar mouse carotid artery ligation model (Kumar & Lindner, 1997; Godin *et al.*, 2000). Vessels were collected after 28 days and subject to functional and morphometric evaluation.

3.2.3 *Myography*

Vascular function was investigated by isometric wire myography (section 2.2) utilising ~2mm long arterial rings cleaned of extraneous peri-adventitial material. For wire-injured vessels, the investigated portion of the femoral artery was that immediately proximal to the femoropopliteal bifurcation. For partial ligation-injured vessels, two vascular rings were isolated from each artery – the femoral artery immediately proximal to the ligated branch, and the popliteal artery immediately distal to the ligated branch. For complete ligation-injured arteries, only the section of femoral artery immediately proximal to the ligature was examined as both popliteal and femoral arteries distal to this point were no longer identifiable. Details of the location of the investigated portions of each vessel are given in **Figure 3-1**. In each case, the equivalent position in the contra-lateral, sham-operated femoral/popliteal artery was examined in parallel as a control. For comparison, additional studies of femoral artery function were performed using mice that were not subject to any prior surgical intervention. In these, femoral artery rings were collected just proximal to femoropopliteal bifurcation from the left and right hind-limb, one of which (randomly selected) was subsequently denuded of endothelium by gentle rubbing of the luminal surface with a human hair. All vessels were mounted across parallel intra-luminal wires (40µm diameter), bathed in physiological salt solution (PSS) at 37°C and a baseline tension of 8mN gradually applied – a value determined by pervious normalisation studies in this laboratory and consistent with those in the literature (Widmer *et al.*, 2006; Bhattacharya *et al.*, 2008).

In every vessel the response to 125mM KCl, iso-osmolar PSS (KPSS) was assessed in triplicate to rejuvenate tissue responsiveness and to provide reference basal contractility of each vascular ring. Cumulative concentration-response curves were then recorded, in sequence, to phenylephrine (PE; 10^{-9}-10^{-4}M; 2 min addition cycle), acetylcholine (ACh; 10^{-9}-$3x10^{-5}$M; 1 min addition cycle), KPSS ($5x10^{-3}$-$1.25x10^{-1}$M;

2 min addition cycle), sodium nitroprusside (SNP; 10^{-9}-10^{-5}M; 1 min addition cycle) and endothelin-1 (ET-1; 10^{-11}-3x10^{-7}M; 3 min addition cycle). Tissues were thoroughly washed with PSS between agonists. Responses to vasodilators (ACh, SNP) were measured in vessels pre-contracted with PE to 80% of the maximal response to this agent. The only exception to this protocol was that, in endothelium-intact and -denuded femoral arteries from un-operated mice, the concentration-response relationship to KPSS and SNP was not determined. At the conclusion of each functional experiment, vessels subject to partial or complete ligation were carefully removed from the myograph and immersed in formalin for 24 hours to allow subsequent histological analysis of lesion formation.

3.2.4 *Histology*

Fixed, ligation-injured arteries were dehydrated and infiltrated with wax, using an automated vacuum infiltration processer, before embedding in a paraffin wax block for microtomy to produce transverse sections. From each vascular ring, 3µm thick sequential sections were cut in a repeating pattern, wherein 15 sections were retained and mounted on 5 electrostatically-charged microscope slides (3 on each), and the subsequent 100µm of tissue discarded, until the entire artery had been traversed. One slide from each level was stained according the United States trichrome method (section 2.4.2) to highlight the elastic laminae and allow delineation of media and neointima. The slide containing the largest neointimal lesion from each vessel was identified and the cross-sectional area of neointima (area of tissue within the internal elastic lamina) and media (area of tissue lying between internal and external elastic laminae) measured from photomicrographs using Photoshop CS3 Extended software (Adobe Systems Inc, USA). Luminal measurements were not recorded as the prior functional investigations in these arteries prevented *in vivo* perfusion fixation.

3.2.5 *Immunohistochemistry*

In sections adjacent to those bearing the largest neointimal lesion for each vessel, medial composition was assessed using immunohistochemistry for α-smooth muscle actin (αSMA) according to the indirect labelled streptavidin biotin method (section

2.4.6). Antibody binding was revealed by the peroxidase-catalysed development of 3,3'-diaminobenzidine (DAB) to an insoluble brown product. Immunoreactive area was measured from photomicrographs using a semi-automated colour de-convolution process and the absolute area in which DAB product was present was expressed as a percentage of the total medial area.

3.2.6 *OPT*

OPT was used to acquire 3D images of uninjured femoral arteries, and those subject to wire-injury, partial ligation-injury and complete ligation-injury procedures. Arteries in which functional investigations were performed were not subject to OPT scanning. Instead, un-injured, 28 day wire-injured and 28 day partial ligation-injured femoral arteries were produced specifically for this purpose (n=2 per injury) by the same methods outlined above (see sections 3.2.2 and 2.3.1), and combined with data from wire- and complete ligation-injured arteries primarily produced for the experiments described in chapters 4 and 5. For the sake of simplicity, these data relating to the description and validation of the OPT technique are presented as one, in this chapter. Regardless of the source of the artery, imaging and analysis was conducted according to the following method. Perfusion fixed femoral arteries were embedded in low melting point agarose, bonded to a magnetic OPT mount, de-hydrated in methanol (>24 hours) and made translucent by immersion in a refractive index matching solution (benzyl alcohol:benzyl benzoate; >24 hours). Vessels were individually scanned for emission (475nm low pass filter) after excitation at 425nm (with 40nm band pass filter) using a Bioptonics 3001 optical projection tomograph. 1.048Mpixel projection images were captured at 0.9° rotation increments and reconstructed by Hamming-filtered back-projection in NRecon software. From these, the position of the intima/media border was manually traced for every 50^{th} scan line, and the position interpolated (and corrected, where necessary) for the interleaved regions. The neointima/lumen border was delineated by grey-level segmentation. Absolute neointimal and luminal sizes were expressed as total volume or 2D area for each scan line. Cross-sectional profiles were produced by plotting 2D lesion and luminal areas (mean of 20 scan lines) against distance of scan line from site of

ligation (ligation-injured vessels) or the femoropopliteal bifurcation (uninjured and wire-injured vessels).

For the comparison of OPT and histological measures, femoral arteries were removed from OPT mounts, washed in methanol (>24 hours) and processed for histology as described in section 3.2.4. 3μm thick serial sections were stained using the United States trichrome method and the regions depicted in a subset of these were located in the reconstructed OPT tomograms of the same vessels. At these points, measurements of 2D neointimal and luminal areas were recorded both from OPT scans and from histological sections. It was not possible to truly blind this comparison, as the poor accuracy of z-axis position information in serial sections required their location within the length of artery, in part, by use of visual cues, such as vessel conformation or the position of arterial branches and ligatures.

3.2.7 *Statistics and data analysis*

For myography data, force developed to vasoconstrictor agents was expressed as absolute value per unit length of vessel ($mN.mm^{-1}$) and as a percentage of the maximal contraction elicited in that vessel by the 3rd initial application of KPSS. Responses to vasodilator agents were expressed as a percentage of the tension existing immediately prior to the addition of the tested agent. Myograpy-generated concentration-response curves were analysed by two-way ANOVA between injured and sham, or endothelium-intact and -denuded pairs. Summary data were generated by least-squares fit of raw data to a logistic equation (chapter 2). Myography summary data, vascular morphometry and composition were tested by Student's un-paired t-test. Comparisons between OPT and histological methods were performed by linear regression, and subsequent F-testing to determine if the slope deviates from a hypothetical value of 1.0. Statistical analysis of all data was performed using Prism 4.0 software (GraphPad software, USA).

3.3 RESULTS

3.3.1 *Physiological parameters*

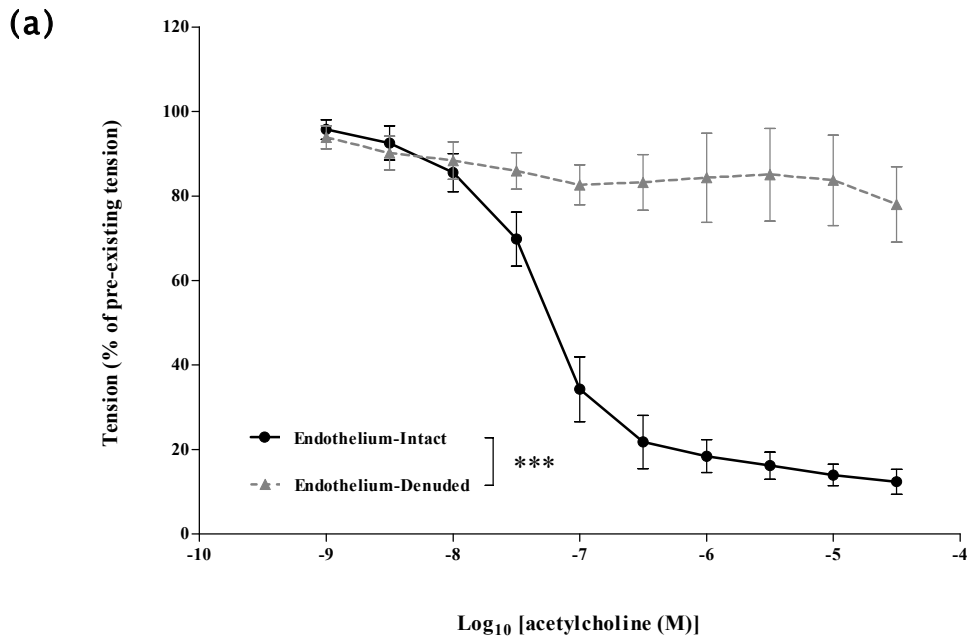
Neither femoral artery wire-injury, nor partial/complete ligation procedures was obviously detrimental to animal health. No post-operative deaths or infections occurred, and no harm was apparent to the limb supplied by the ligated femoral artery. In agreement, no abnormalities of post-operative body weight change were observed (**Table 3-1**, page 108).

3.3.2 *Vasomotor function in uninjured femoral arteries*

In un-injured femoral arteries, the α_1-adrenoceptor agonist phenylephrine stimulated concentration-dependent vasoconstriction. Maximum responses to this agent, expressed as % of contraction to KPSS to normalise for the medial damage implicit in mechanical endothelial denudation, was significantly enhanced by removal of the endothelium (**Table 3-2**, page 109). Endothelial denudation also abolished the otherwise robust vasodilator response to acetylcholine in phenylephrine pre-contracted femoral artery rings (**Figure 3-2**). ET-1 potently elicited vasoconstriction in intact and denuded vessels (**Figure 3-2**). Two-way ANOVA indicated that this response was enhanced by endothelial removal, but changes in pD_2 and E_{max} (% KPSS) did not reach significance.

3.3.3 *Vasomotor function in wire-injured femoral arteries*

Wire-injured (14 days) femoral arteries failed completely to contract to either membrane depolarisation (KPSS) or α_1-adrenoceptor stimulation (phenylephrine). In contrast, un-injured contra-lateral femoral arteries from the same mice, tested in parallel to their injured counterparts, robustly responded to both agents in the expected manner (**Figure 3-3**). After this striking pattern had been observed in injured/un-injured femoral artery pairs from 2 animals, further attempts were abandoned and the remaining study animals used for an alternative purpose.



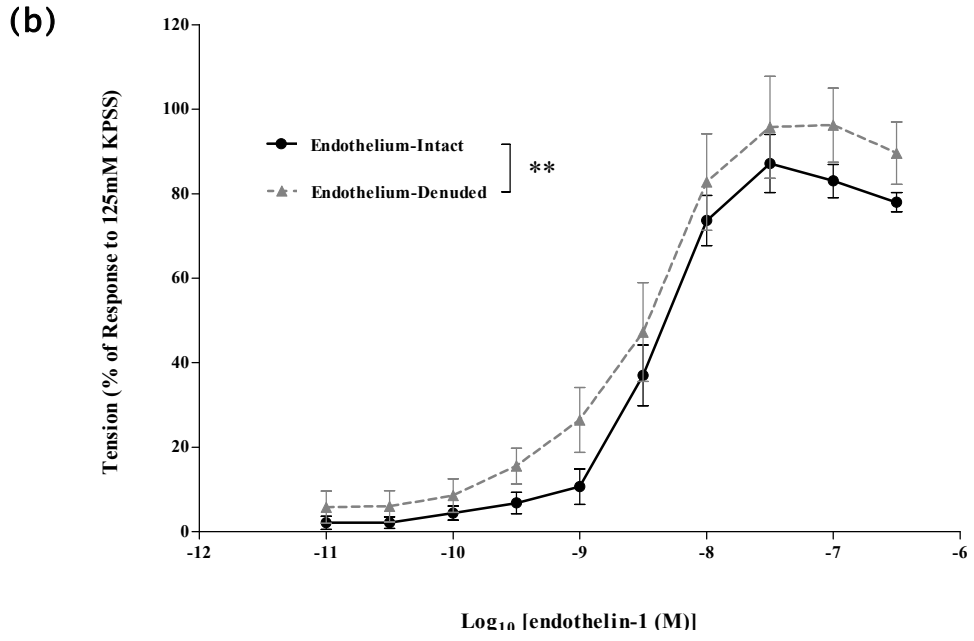


Figure 3-2 Impact of endothelial denudation on the vasomotor response to acetylcholine and ET-1.

Femoral artery rings were examined by wire myography. Acetylcholine elicited relaxation of pre-contracted rings, and this was abolished by removal of the endothelium (a). Endothelial denudation also modestly sensitised vessels to the contractile effect of ET-1 (b). n=7-9. Data are mean ± SEM. **, $p<0.01$; ***, $p<0.001$ by two-way ANOVA.

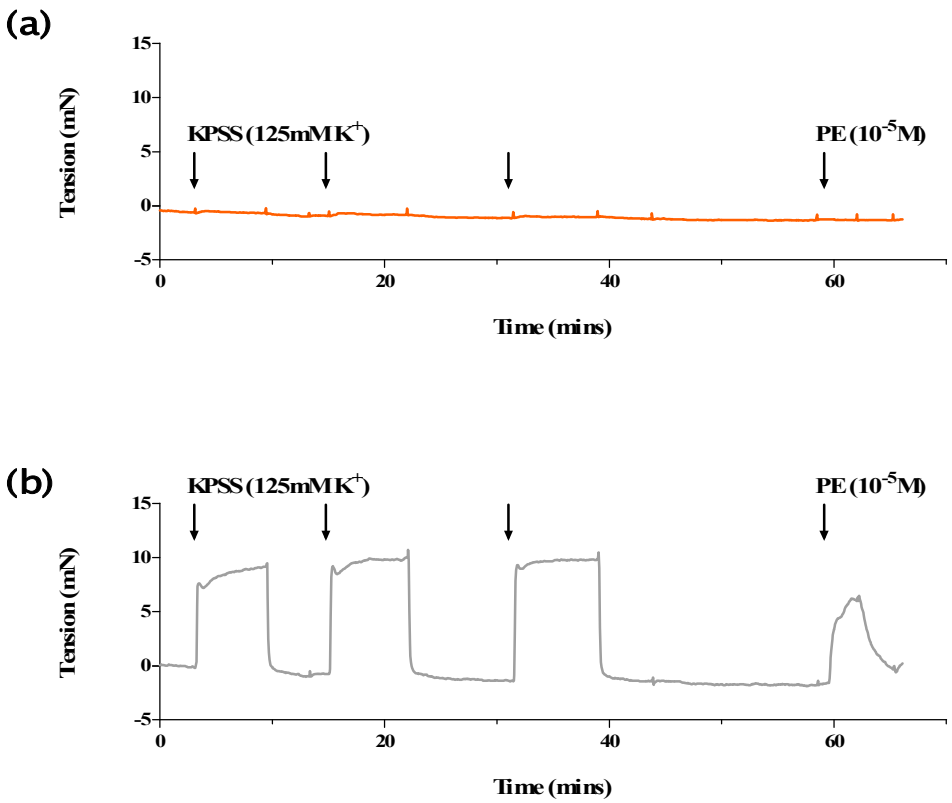


Figure 3-3 Impact of femoral artery wire-injury on vasomotor response.

Vascular function of wire-injury femoral arteries was investigated by wire myography, 14 days post-injury. Representative tension traces of the response of thus injured vessels (a) and contra-lateral uninjured control vessels (b) to membrane depolarization (KPSS, K^+ 125mM Na^+ 0mM) and the α_1-adrenoreceptor agonist, phenylephrine (PE; 10^{-5}M) are shown. 14 day wire-injured vessels completely failed to response to either stimuli, in contrast to robust responses to both in uninjured control vessels. Traces are representative of 2 experiments.

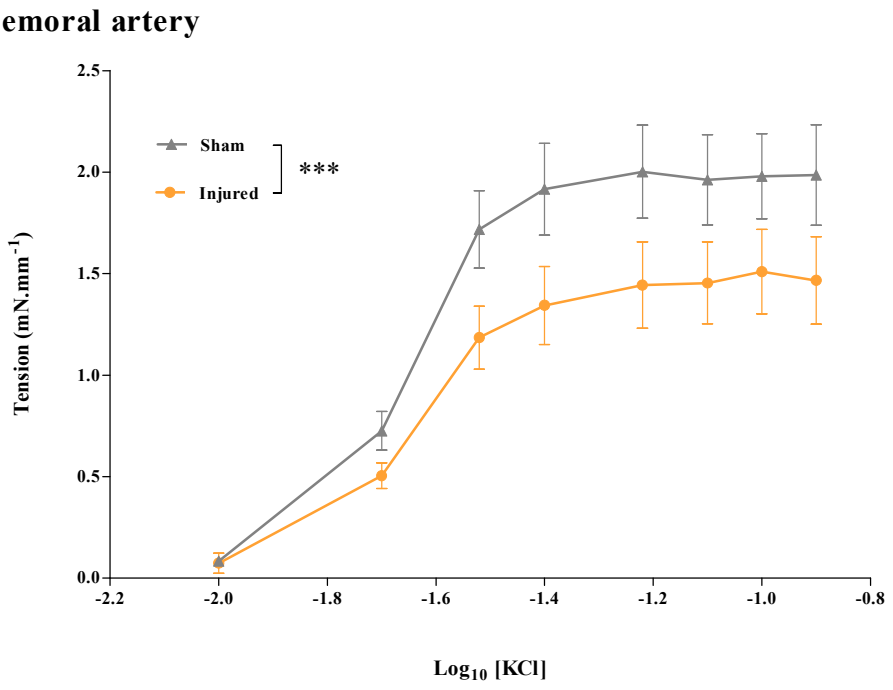
3.3.4 *Partial ligation injury*

3.3.4.1 *Vasomotor function*

Although not the basis of statistical testing, several differences in character were apparent between femoral and popliteal artery rings. Absolute ($mN.mm^{-1}$) E_{max} values to tested vasoconstrictors, were, in general, substantially lower in the popliteal than the femoral artery (**Table 3-3**, page 110). The response to endothelin-1 also appeared to by bi-phasic in popliteal arteries and monophasic in femoral arteries. Insufficient data points were acquired to allow fitting of these to a bi-phasic logistic equation, however, and thus summary data were produced by best-fit of a monophasic relationship.

In contrast to wire-injured femoral arteries, all vessels subject to partial ligation-injury demonstrated contractile responses to tested substances. Indeed, partial ligation was associated with little to no defects in vasomotor responsiveness in the popliteal artery. In adjacent femoral artery rings however, clear defects in vascular function were evident. Absolute ($mN.mm^{-1}$) responses to both KPSS (**Figure 3-4**) and ET-1 were significantly impaired by injury in femoral (both $p<0.0001$ by two-way ANOVA) but not popliteal artery rings (KPSS $p=0.38$; ET-1 $p=0.99$). In both cases, however, neither changes in pD_2 nor E_{max} reached statistical significance (**Table 3-3**, page 110). The absolute ($mN.mm^{-1}$) vasoconstrictor response to phenylephrine was also not different in either the femoral or popliteal artery between injured and sham groups (**Table 3-3**, page 110).

Endothelium-dependent vasodilator responses were also altered. Acetylcholine-stimulated relaxation of phenylephrine pre-contracted femoral (but not popliteal) artery rings (**Figure 3-5**), was significantly impaired in partial ligation injured, manifesting as reduced sensitivity to this agent (lower $-\log(EC_{50})$; $p=0.013$; **Table 3-3**, page 110). No concurrent changes in endothelium-independent vasodilatation to sodium nitroprusside were apparent in either injured femoral or popliteal rings (**Table 3-3**, page 110).



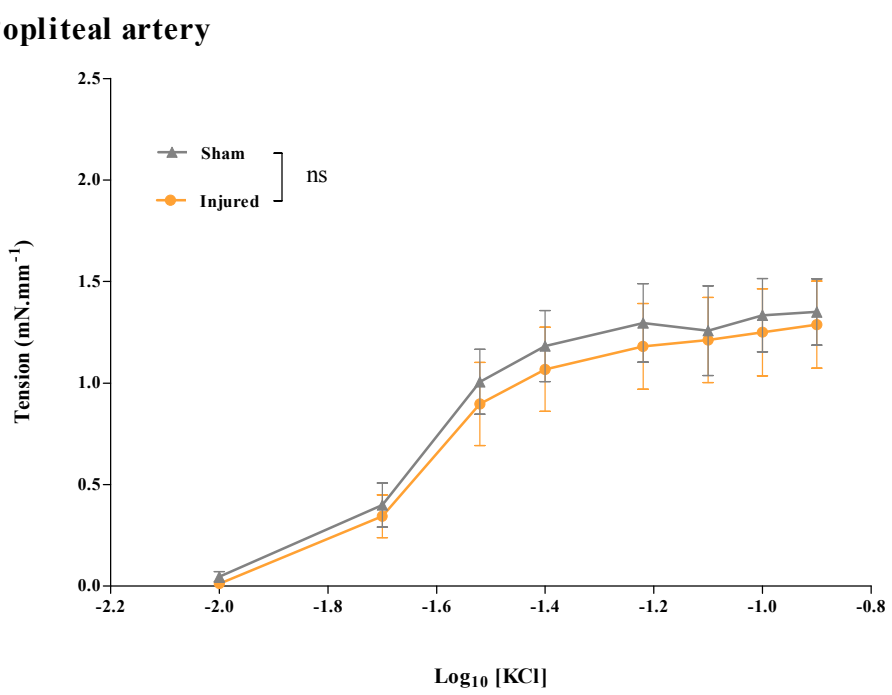
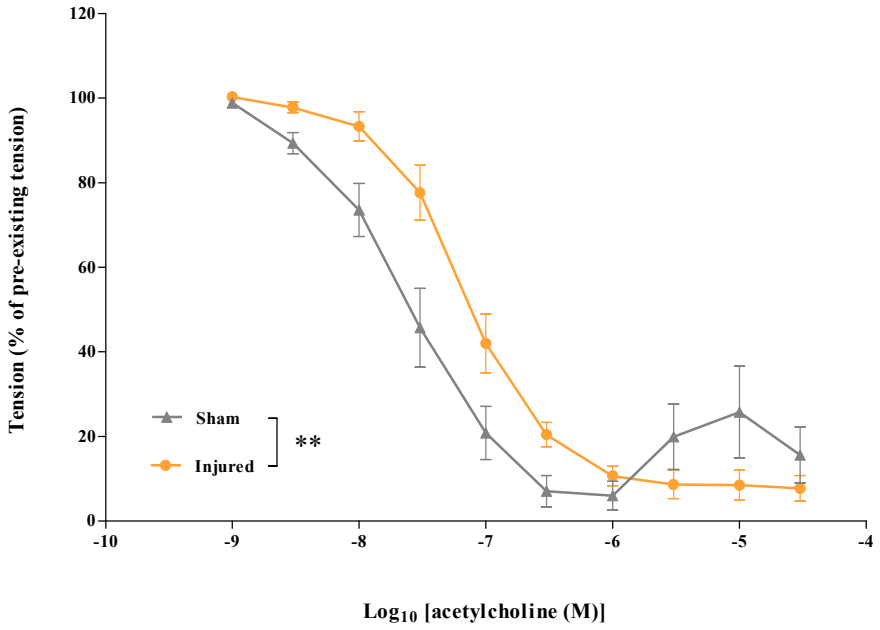


Figure 3-4 Impact of partial femoral artery ligation on vasoconstrictor response to potassium in femoral and popliteal artery rings.

Femoral arteries were ligated just distal to the femoropopliteal bifurcation. 28 days later, vascular function was studied in the proximal femoral artery (a) and popliteal artery (b) by wire myography. Injured and uninjured vessels from both locations contracted strongly to potassium. In the femoral (a) but not popliteal artery (b), this response was significantly impaired with partial ligation injury, as compared to sham operated contralateral controls. n=5-6. Data are mean ± SEM. ns, $p>0.05$; ***, $p<0.001$ by two-way ANOVA.

(a) Femoral artery



(b) Popliteal artery

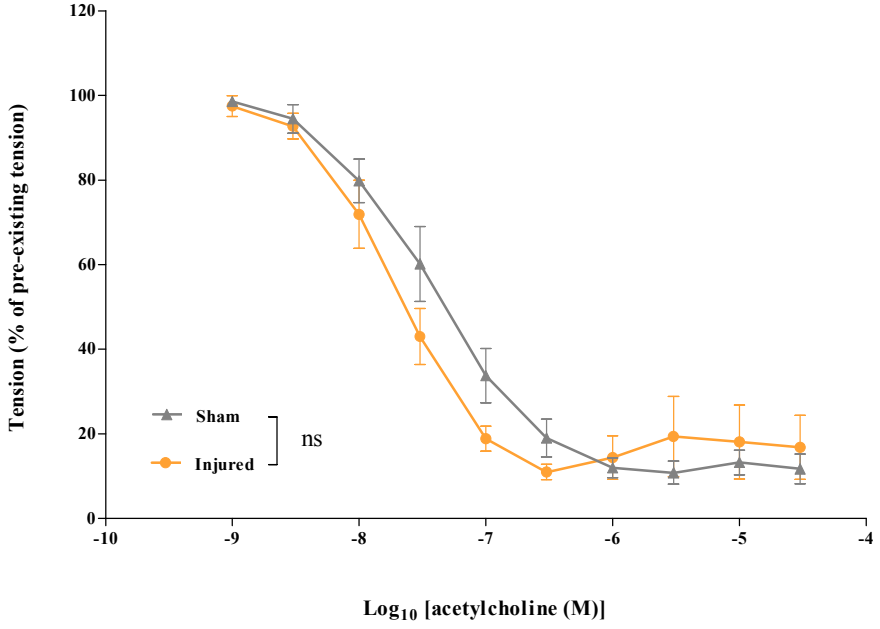


Figure 3-5 Impact of partial femoral artery ligation on vasodilator response to acetylcholine in femoral and popliteal artery rings.

Femoral arteries were ligated just distal to the femoropopliteal bifurcation. 28 days later, vascular function was studied in the proximal femoral artery (a) and popliteal artery (b) by wire myography. Phenylephrine pre-contracted vessels from both locations dose-dependently relaxed in response to acetylcholine. In the femoral (a) but not popliteal artery (b), this response was significantly impaired (parallel rightward shift) by partial ligation injury, as compared to sham operated contra-lateral controls. n=5-6. Data are mean ± SEM. ns, $p>0.05$; **, $p<0.01$ by two-way ANOVA.

3.3.4.3 *Morphology and composition*

Following functional investigations, injured and sham femoral artery rings from mice subject to the partial ligation procedure were examined for morphological alterations (**Figure 3-6**). Popliteal artery rings, in which no functional disturbances were observed, were not analysed further. Most partially-ligated femoral artery rings contained some neointimal lesion although this was typically extremely small and focal. Indeed, neointimal lesion size was not significantly different to that in sham operated rings, which also contained occasional, small neointimal lesions as a result of peri-vascular manipulations (**Figure 3-7**). Medial area was also not altered by partial ligation-injury and consequently, neither was intima/media ratio (**Table 3-4**, page 111). αSMA immunoreactivity was observed throughout the media in both groups (**Figure 3-6**), although the fractional area of this was not different between injured and sham-operated control vessels (**Figure 3-7**).

3.3.5 *Complete ligation injury*

3.3.5.1 *Vasomotor function*

Functional disturbances in complete ligation-injured femoral arteries were broadly similar to those seen in the proximal femoral artery following partial ligation. Absolute ($mN.mm^{-1}$) contractions induced by phenylephrine (**Table 3-4**, page 111) and KPSS (**Figure 3-8**) were significantly impaired in injured vessels as compared to sham (PE $p=0.006$; KPSS $p<0.0001$). A trend ($p=0.06$) towards similarly reduced responsiveness to ET-1 was also apparent (**Figure 3-8**). For KPSS only, this was associated with a reduction in E_{max} ($p=0.029$; **Table 3-4**, page 111). Although the response to ET-1 was not significantly different between sham-operated and complete ligation-injured arteries, it was apparent that the responses elicited in both were dramatically lower than those that would be expected. Indeed, these values (sham $E_{max}=0.53 \pm 0.13 mN.mm^{-1}$, complete ligation $E_{max}=0.46 \pm 0.09\ mN.mm^{-1}$) were lower than those determined for the response to ET-1 any other studies in this thesis, including femoral arteries from un-operated mice of the same age and strain ($E_{max}=2.43 \pm 0.23\ mN.mm^{-1}$).

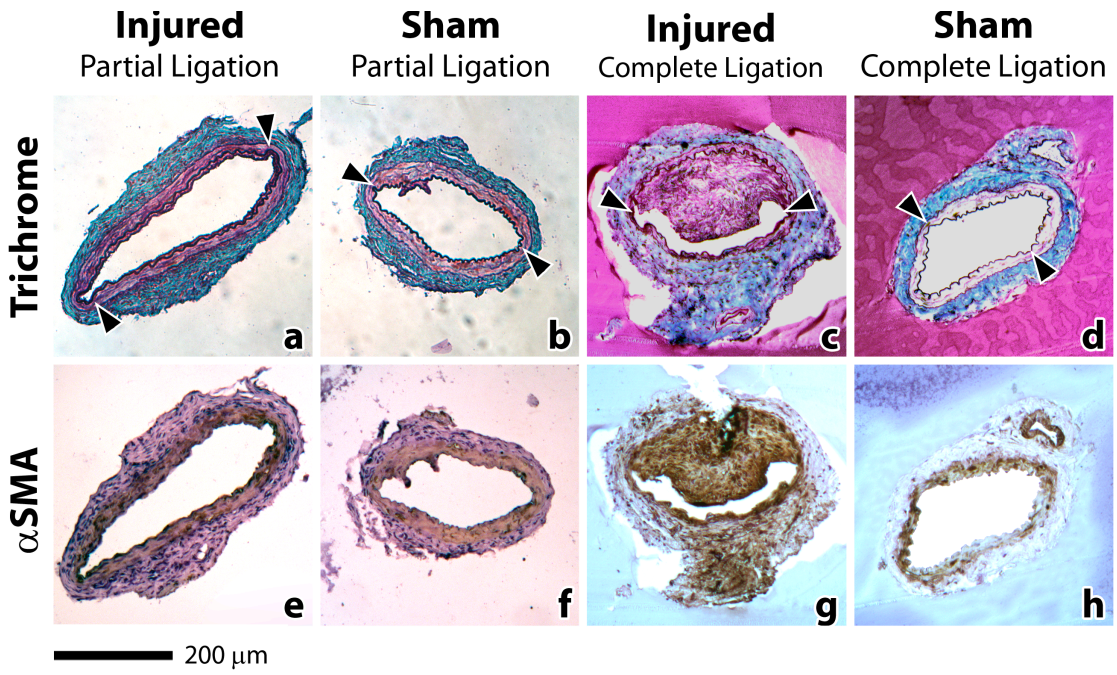


Figure 3-6 Impact of partial and complete femoral artery ligation on vascular structure.

Femoral arteries were ligated just distal (partial ligation) or across (complete ligation) the femoropopliteal bifurcation and allowed to respond for 28 days. After functional investigation, vessels were histologically examined. Representative photomicrographs for these vessels stained by United States Trichrome (a-d) or immunohistochemistry for α-smooth muscle actin are shown. In all, probable sites of damage by myography mounting are indicated with arrowheads. Partial ligation-injured vessels were similar in appearance to their sham operated controls with only minor evidence of neointima formation (a-b). αSMA immunoreactivty was present throughout the media and was similar between injured and sham vessels (e-f). Complete ligation-injured vessels contained large neointimal lesions, in contrast to sham-operated vessels (c-d). αSMA immunoreactivity was again present throughout the media and in the neointima of injured vessels (g-h). αSMA, alpha smooth muscle actin.

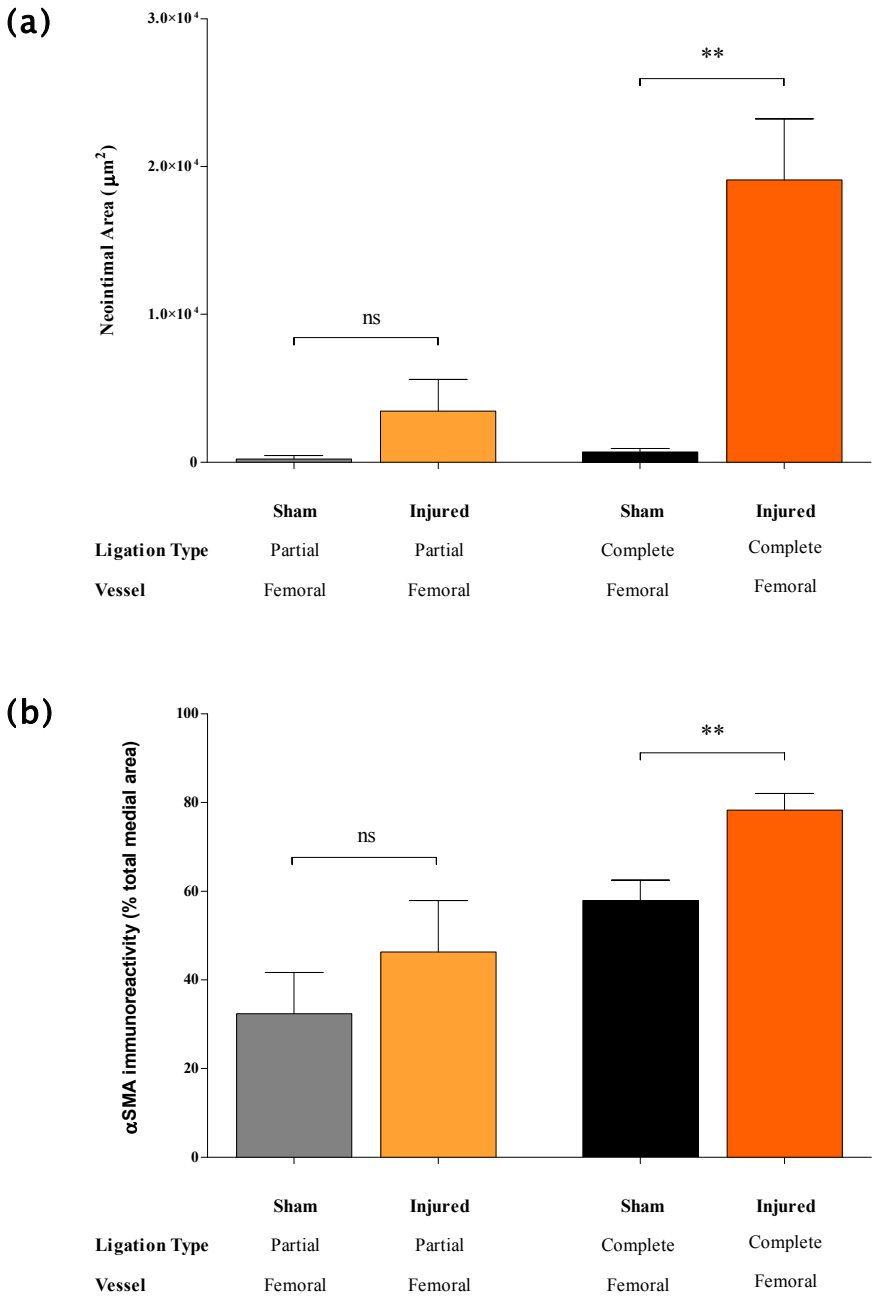


Figure 3-7 Impact of partial and complete femoral artery ligation on neointimal lesion size and medial composition.

Femoral arteries were ligated just distal (partial ligation) or across (complete ligation) the femoropopliteal bifurcation and allowed to respond for 28 days before functional and histological examination. United States Trichrome stained sections indicated some evidence of neointima formation in some vessels from all groups. Maximum lesion size in partial ligation-injured vessels was not different from sham-operated controls. In contrast, complete ligation-injury stimulated formation of much larger lesions (a). αSMA immunoreactivity was present throughout the media in all groups. The areas of this did not differ between partial ligation and sham-operated vessels but were significantly greater in complete ligation-injured vessels than their controls (b). n=3-6. Data are mean ± SEM. ⟨SMA, ⟨–smooth muscle actin; ns, $p>0.05$; **, $p<0.01$ by Student's unpaired t-test.

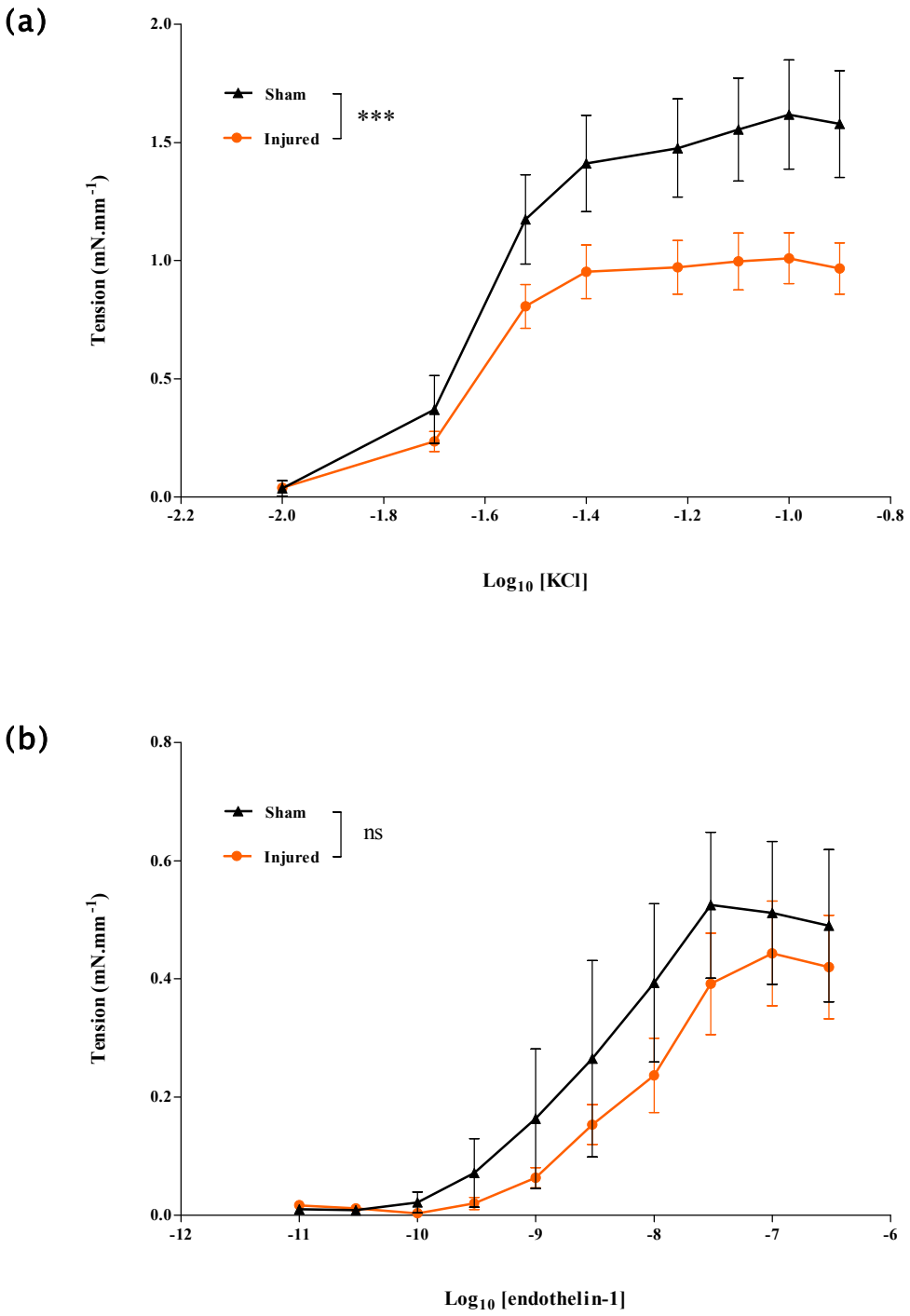


Figure 3-8 Impact of complete femoral artery ligation on vasoconstrictor responses to potassium and ET-1.
Femoral arteries were ligated across the femoropopliteal bifurcation. 28 days later, vascular function was studied in the proximal femoral artery by wire myography. Injured and uninjured vessels contracted strongly to potassium (a) and ET-1 (b). Complete ligation was associated with a significant impairment of the vasoconstrictor response to potassium as compared to sham-operated controls (a). A trend towards reduced responsiveness to ET-1 in injured vessels was not significant (b; p=0.06). n=6. Data are mean ± SEM. ET-1, endothelin-1; ns, p>0.05; ***, p<0.001 by two-way ANOVA.

Endothelium-dependent vasodilatation stimulated by acetylcholine (**Figure 3-9**) was impaired in injured vessels (p=0.001 by two-way ANOVA). This appeared to be a reduction in maxium response, but neither changes in E_{max} nor $-\log(EC_{50})$ reached significance (**Table 3-5**, page 112). The endothelium-independent relaxation elicited by sodium nitroprusside (**Figure 3-9**) was also reduced by complete ligation injury ($p<0.0001$) and this was associated with a decrease in E_{max} (p=0.046; **Table 3-5**, page 112).

3.3.5.2 *Morphology and composition*

Unlike partial ligation-injured and sham-operated control vessels, those subject to complete ligation had the gross appearance of lesion formation and indeed, were substantially more difficult to mount in the myograph owing to luminal stenosis. Analysis of vessel morphology revealed the presence of large extracellular matrix- and smooth muscle-rich neointimal lesions in complete ligation-injured femoral artery rings (**Figure 3-6**). These were significantly larger than the small, occasional neointimal lesions formed in sham-operated control vessels (p=0.001; **Figure 3-7**). Medial cross-sectional area was not different between injured and sham vessels (p=0.72; **Table 3-4**, page 111) and therefore, intima/media ratio was increased relative to sham vessels (p=0.004; **Table 3-4**, page 111). Whilst these lesions were apparent in every vascular ring from injured arteries they did not typically extend the full length of segment of artery isolated for functional analysis. αSMA immunoreactivity was present throughout the media both in injured and in sham vessels and was also abundant in the neointima of injured vessels (**Figure 3-6**). The fractional area of this immunoreactivity was significantly greater in the media of complete ligation-injured vessels than in those subject to the sham procedure (p=0.007; **Figure 3-7**).

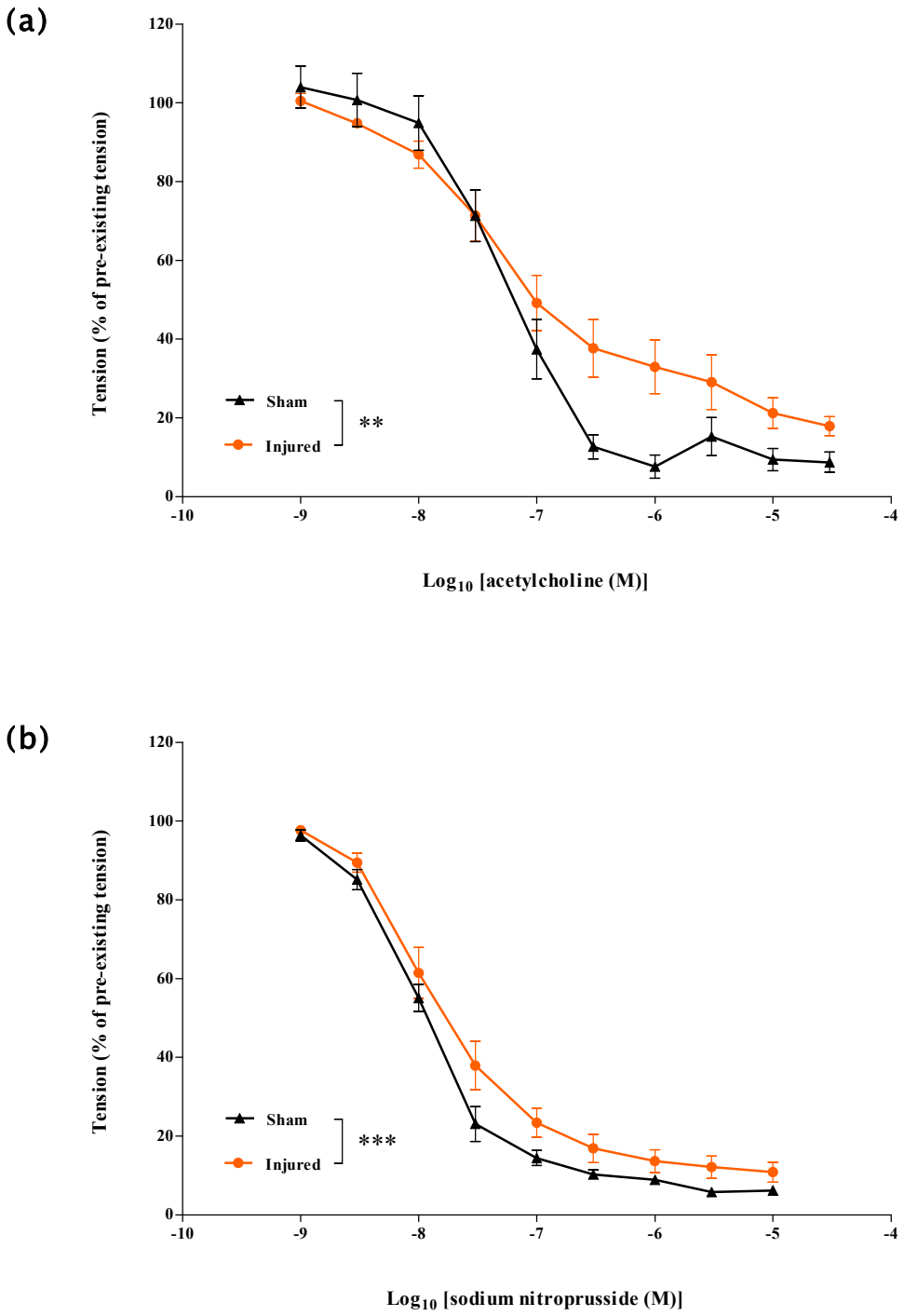


Figure 3-9 Impact of complete femoral artery ligation on vasodilator responses to acetylcholine and sodium nitroprusside.

Femoral arteries were ligated across the femoropopliteal bifurcation. 28 days later, vascular function was studied in the proximal femoral artery by wire myography. Phenylephrine pre-contracted, injured and uninjured vessels dose-dependently relaxed in response to both acetylcholine (a) and sodium nitroprusside (b). Complete ligation was associated with a significant impairment of the vasodilator response to both agents as compared to sham-operated controls. n=6. Data are mean ± SEM. **, $p<0.01$; ***, $p<0.001$ by two-way ANOVA.

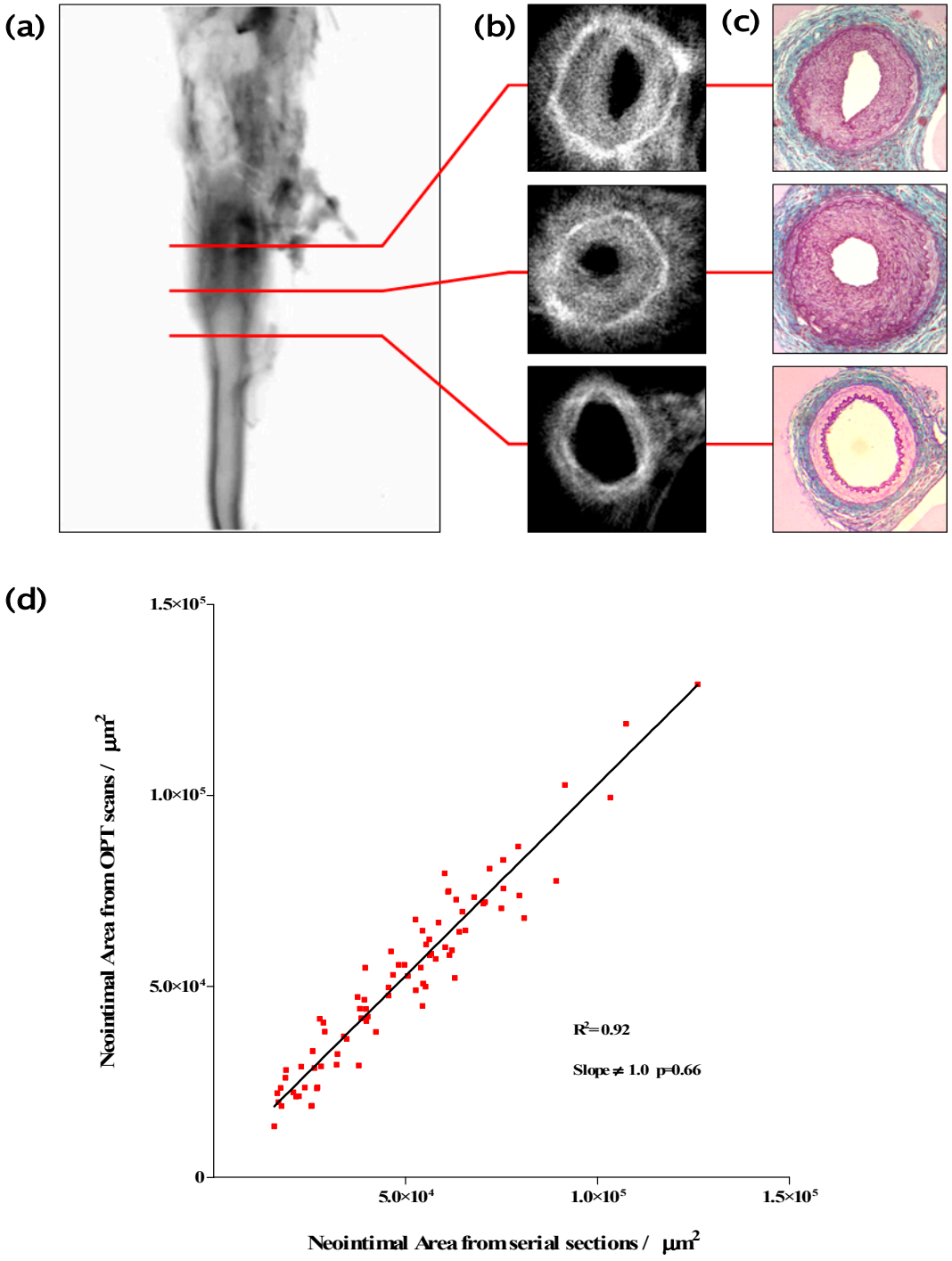


Figure 3-10 Comparison of OPT and histological methods for 2D analysis of lesion size.

Wire and ligation injured femoral arteries were subject to perfusion fixation and optical projection tomographic scanning, 28 days post-injury, before histological analysis of the same vessels. Neointimal lesions were clearly visible in both projection images (a) and in re-constructed virtual sections (b). In the latter, media, neointima and lesion could be clearly distinguished by the difference in auto-fluorescence from these layers. These possessed striking similarity to histological, United States Trichrome stained sections of the same regions of the same vessels (c). Comparison of 2D measures of neointimal area derived from OPT scans and histological sections of the same area by linear regression showed a strong correlation and that the slope of relationship did not differ from 1 (d). n=82. OPT, optical projection tomography.

3.3.6 *Optical projection tomography (OPT)*

3.3.6.1 *Comparison with histology*

OPT imaging of femoral arteries proved very successful. Arterial structure was clearly visible both in raw projection images, and back-projected reconstructions (**Figure 3-10**). In injured vessels, neointimal lesion-like structures were identifiable within the lumen, and could be delineated from the media and lumen by the stronger auto-fluorescent signal emitted by the former, and the absence of signal from the latter. Furthermore, tomographically reconstructed cross-sections possessed a striking resemblance to equivalent United States trichrome stained histological sections cut from the same vessels.

As such, it was possible to develop methods for quantitative measurement of neointimal and luminal dimensions from OPT re-constructions. To validate these, cross-sectional areas of neointima and lumen were determined from serial histological sections and corresponding 2D planes within OPT reconstructions of the same vessels. For both neointimal and luminal areas, strong and significant correlations (R^2 = 0.92, R^2 = 0.74 respectively) were present by linear regression between the two measurement modalities (**Figure 3-10**). Importantly, in each case, the slope of the relationship between histological and OPT measurements did not significantly deviate from 1.0, and intercepted the y-axis close to 0.

3.3.6.2 *Application to comparison of injury models*

OPT reconstructions of separate vessels were used to visualise the extent of lesion formation in partial and complete ligation models along the length of vessels. Lesion profiles were produced by plotting neointimal lesion size against distance proximal to femoropopliteal bifurcation (**Figure 3-11**). These confirmed that, in the partial ligation model, any neointimal thickening that occurs is restricted to the immediate vicinity of the ligature with little impact on luminal dimensions. Indeed, lesion profiles from partial ligation-injured vessels were similar to those of uninjured femoral arteries. In complete ligation-injured arteries, an occlusive neointima was

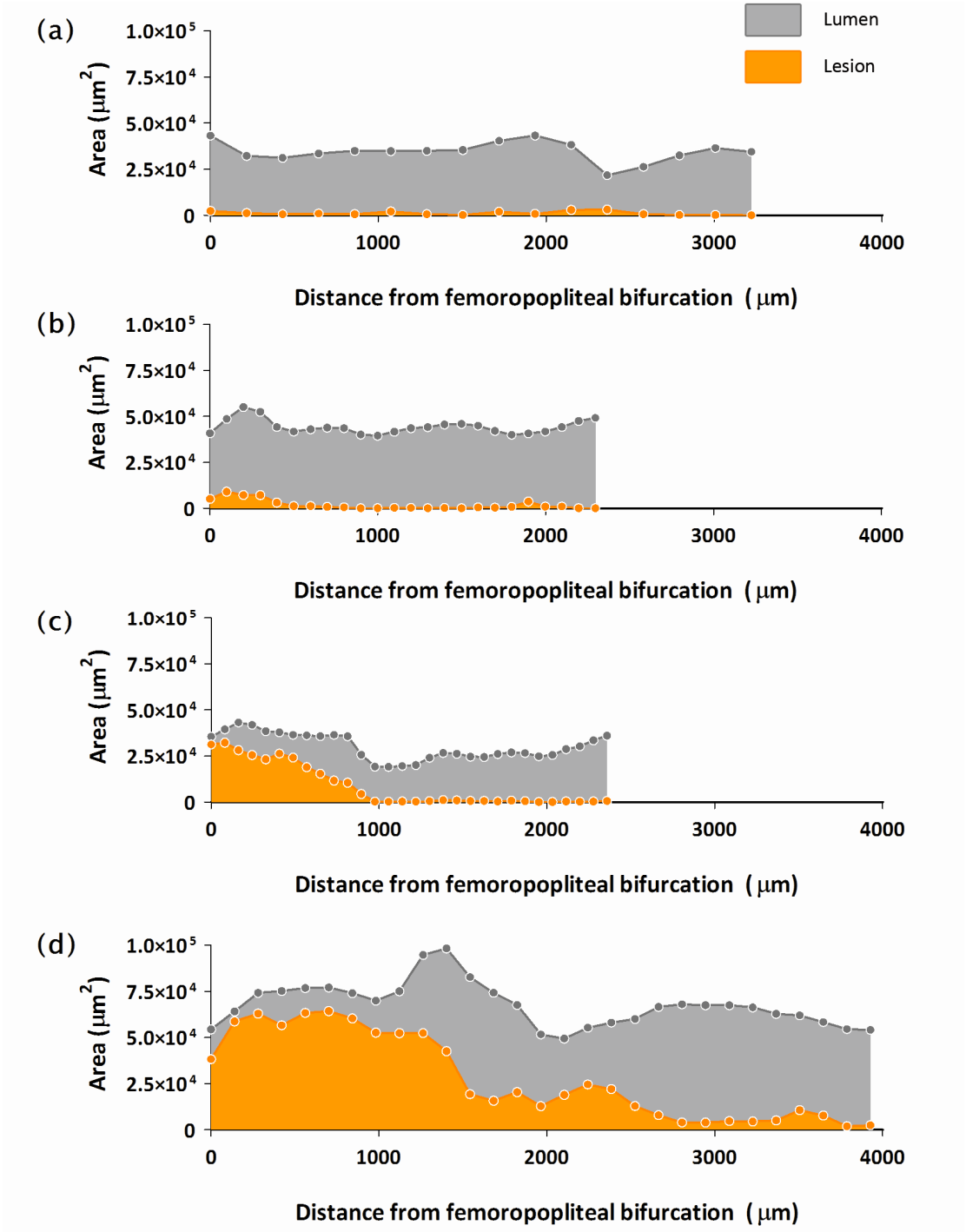


Figure 3-11 Impact of femoral artery wire and ligation injury on the axial distribution of neointima formation. Uninjured (a), partially (b) or completely ligated (c) and wire-injured femoral arteries (d) were subject to optical projection tomographic scanning, 28 days post-injury. Volumes corresponding to neointima and lumen were defined in reconstructed tomograms. From these, neointimal (grey) and lumenal area (orange) were plotted against distance from the femoropopliteal bifurcation or ligation. Almost no neointima was detected in uninjured vessels (a). In partial ligation vessels, lesion formation was minimal and restricted to the immediate vicinity of ligature (b). Complete ligation elicited formation of lesions of moderate size and length (c). Wire-injury induced formation of large lesions that extend proximally much further than those induced by ligation (d). Profiles are representative of at least 2 vessels.

seen proximal to the site of ligation and this gradually transitioned to morphological normal vessel over a moderate distance. For comparison, profiles of wire-injured vessels indicated lesions of the largest size and which extended the furthest down the vessel length and were associated with outward remodelling (i.e. increased lumen+neointimal cross-sectional area).

Study Group	Wire Injury	Partial Ligation	Complete Ligation
N	2	6	6
Body weight at study start (**g**)	25.10 ± 0.10	26.27 ± 0.72	22.43 ± 0.82
Body weight at study end (**g**)	26.15 ± 0.75	29.97 ± 1.37	25.60 ± 1.04
Body weight change (**g**)	1.05 ± 0.85	3.42 ± 0.73	3.17 ± 0.35

Table 3-1 The impact of femoral artery injury models on post-operative weight gain.

All studied animals gain weight normally following femoral artery wire- and ligation-injury procedures. The lesser increase in body weight observed following wire- than ligation- injury reflects the shorter period of post-operative recovery (14 vs. 28 days). Data are mean ± SEM.

Procedure		**Uninjured**	**Uninjured**
Endothelium denuded?		No	Yes
PE	n	9	7
	pD_2	5.64 ± 0.11	5.83 ± 0.10
	E_{max} (%)	**66.3 ± 7.1**	**84.8 ± 4.6 ***
	E_{max} ($mN.mm^{-1}$)	1.72 ± 0.27	1.26 ± 0.26
ACh	n	9	7
	$-log(EC_{50})$	7.25 ± 0.19	-
	E_{max} (%)	87.3 ± 3.5	-
ET-1	n	7	7
	pD_2	8.48 ± 0.10	8.59 ± 0.22
	E_{max} (%)	83.9 ± 3.9	101.3 ± 9.2
	E_{max} ($mN.mm^{-1}$)	**2.43 ± 0.23**	**1.36 ± 0.38 ***

Table 3-2 Impact of endothelial denudation on femoral artery vasomotor function.

Femoral artery vascular function was investigated by wire myography. Removal of the endothelium almost abolished the vasodilator response to ACh and potentiated constrictor responses to PE and ET-1 when expressed as % of response to KPSS, but reduced absolute contractility. n=7-9. Data are mean ± SEM. PE, phenylephrine; ACh, acetylcholine; ET-1, endothelin-1; KPSS, high potassium physiological salt solution; *, $p<0.05$ by Student's unpaired t-test between endothelium-intact and endothelium-denuded vessels.

Procedure		**Injured**	**Sham**	**Injured**	**Sham**
Ligation type?		Partial		Partial	
Ring Location?		Femoral	Femoral	Popliteal	Popliteal
PE	n	6	5	6	5
	pD_2	6.21 ± 0.10	5.96 ± 0.05	6.01 ± 0.07	6.22 ± 0.13
	E_{max} ($mN.mm^{-1}$)	1.11 ± 0.15	1.43 ± 0.17	0.82 ± 0.18	0.87 ± 0.18
KPSS	n	6	5	6	5
	pD_2	1.67 ± 0.02	1.67 ± 0.02	1.59 ± 0.03	1.63 ± 0.02
	E_{max} ($mN.mm^{-1}$)	1.48 ± 0.21	1.99 ± 0.22	1.24 ± 0.21	1.32 ± 0.18
ACh	n	6	5	6	5
	-log(EC_{50})	**7.20 ± 0.10**	**7.70 ± 0.13 ***	7.78 ± 0.10	7.40 ± 0.14
	E_{max} (%)	91.7 ± 3.1	86.3 ± 4.9	84.9 ± 5.9	89.5 ± 2.2
SNP	n	6	5	6	5
	-log(EC_{50})	7.91 ± 0.07	7.96 ± 0.16	7.85 ± 0.10	7.85 ± 0.03
	E_{max} (%)	97.6 ± 0.8	98.0 ± 1.9	96.1 ± 1.9	97.6 ± 0.8
ET-1	n	6	5	6	5
	pD_2	8.53 ± 0.09	8.79 ± 0.30	8.50 ± 0.10	8.43 ± 0.17
	E_{max} ($mN.mm^{-1}$)	1.53 ± 0.18	1.98 ± 0.19	1.15 ± 0.18	1.23 ± 0.14

Table 3-3 Impact of partial femoral artery ligation on vasomotor responses.

Femoral arteries were ligated just distal to the femoropopliteal bifurcation. 28 days later, vascular function was studied in the proximal femoral artery and popliteal artery by wire myography. Partial ligation was associated with numerous changes to vasomotor function in the proximal femoral artery including impaired absolute ($mN.mm^{-1}$) responses to vasoconstrictors, and to the endothelium-dependent vasodilator, acetylcholine. In the adjacent popliteal artery, few changes in responsiveness to these agents were noted. n=5-6. Data are mean ± SEM. PE, phenylephrine; KPSS, high potassium physiological salt solution; ACh, acetylcholine; SNP, sodium nitroprusside; ET-1, endothelin-1; *, $p<0.05$ by Student's unpaired t-test between injured and appropriate sham operated vessels.

Procedure	**Injured**	**Sham**	**Injured**	**Sham**
Ligation type?	Complete		Partial	
(a) Morphometry (Histology)				
n	6	6	3	4
Neointimal area (**x10^{3}µm^2**)	**19.10 ± 4.12**	**0.70 ± 0.24 ***	3.46 ± 2.16	0.22 ± 0.22
Medial area (**x10^{3}µm^2**)	13.68 ± 1.70	14.48 ± 1.38	19.07 ± 1.84	17.97 ± 1.10
Intima/Media ratio	**1.54 ± 0.40**	**0.05 ± 0.02 ***	0.21 ± 0.15	0.01 ± 0.01
(b) Composition (IHC)				
n	6	6	3	4
Intimal αSMA IR (**%**)	78.9 ± 4.2	-	-	-
Medial αSMA IR (**%**)	**78.3 ± 3.8**	**57.9 ± 4.6 ****	46.24 ± 11.62	32.36 ± 9.28

Table 3-4 Impact of partial and complete femoral artery ligation of vascular morphology and composition.
Femoral arteries were ligated just distal (partial ligation) or across (complete ligation) the femoropopliteal bifurcation and allowed to respond for 28 days. After functional investigation, vessels were histologically examined by sectioning and staining with United States Trichrome (a-d) or immunohistochemistry for α-smooth muscle actin. Although some neointima formation was present in all groups, only complete ligation was associated with significantly greater lesion size and intima/media ratio than sham operated control vessels. The fraction of medial area immunoreactive for αSMA was also increased in complete ligation-injured vessels relative to sham. No such change to medial composition was apparent following partial ligation-injury. n=3-6. Data are mean ± SEM. αSMA, α-smooth muscle actin; IR, immunoreactivity. *, $p<0.05$; **, $p<0.01$ by Student's unpaired t-test between injured and appropriate sham operated vessels.

Procedure		**Injured**	**Sham**
Ligation type?		Complete	
	n	6	6
PE	pD_2	5.91 ± 0.08	6.00 ± 0.08
	E_{max} ($mN.mm^{-1}$)	0.57 ± 0.07	0.83 ± 0.21
	n	6	6
KPSS	pD_2	1.63 ± 0.01	1.61 ± 0.02
	E_{max} ($mN.mm^{-1}$)	**0.99 ± 0.11**	**1.56 ± 0.22 ***
	n	6	6
ACh	$-log(EC_{50})$	7.3 ± 0.27	7.35 ± 0.13
	E_{max} (%)	79.4 ± 4.9	90.6 ± 2.7
	n	6	6
SNP	$-log(EC_{50})$	7.91 ± 0.10	8.02 ± 0.07
	E_{max} (%)	**86.3 ± 2.3**	**92.2 ± 1.2 ***
	n	6	6
ET-1	pD_2	8.16 ± 0.11	8.31 ± 0.24
	E_{max} ($mN.mm^{-1}$)	0.46 ± 0.09	0.53 ± 0.13

Table 3-5 Impact of complete femoral artery ligation of vasomotor function.

Femoral arteries were ligated just across (complete ligation) the femoropopliteal bifurcation. 28 days later, vascular function was studied in the proximal femoral artery by wire myography. As with partial ligation, complete ligation resulted in several functional impairments in the femoral artery. Absolute contractility to K+ was significantly reduced, and both endothelium-dependent (acetylcholine) and –independent (SNP) vasodilator responses were attenuated. n=6. Data are mean ± SEM. PE, phenylephrine; KPSS, high potassium physiological salt solution; ACh, acetylcholine; SNP, sodium nitroprusside; ET-1, endothelin-1; *, $p<0.05$ by Student's unpaired t-test between injured and sham operated vessels.

3.4 DISCUSSION

The experiments described in this chapter address the functional and morphological consequences of acute injury to the mouse femoral artery by intra-luminal wire-insertion or ligation of arteries that divide from the femoropopliteal bifurcation. Following wire-injury, all contractile function of femoral rings was lost, reflecting the severity of this insult and preventing any determination of endothelial function. In ligation-injury models functional changes were present but less severe. Both partial and complete ligation of the femoral artery resulted in moderate impairment to contractility and endothelium-dependent vasodilatation. Interestingly, however, partial ligation was associated with only minimal neointimal lesion formation whilst in complete ligation-injured vessels substantial neointimal thickening was present. Analysis of lesion morphology resulting from each injury in three dimensions using the technique of optical projection tomography (OPT) confirmed these observations and demonstrated the potential of this imaging modality in studies of this kind. Lesion profiles generated in this way indicated that partial ligation-injured vessels were barely distinguishable from those without injury, whereas complete ligation resulted in lesions of considerable size. These in turn, however, were smaller in length and cross-sectional area than those resulting from wire-injury. Thus, functional, histological and tomographic analysis of injured femoral arteries has revealed complete but not partial ligation of this vessel to be a robust model of neointima formation associated with more modest lesion growth and deficits to function, and in which, the retention of some endothelial function is certain.

3.4.1 *Femoral artery wire-injury abolishes vascular reactivity*

At 14 days, wire-injured femoral arteries were completely unresponsive to vasoconstrictor substances, preventing subsequent determination of endothelial cell function by these means. This is perhaps unsurprising due to the severity of the medial stretch injury inflicted in this model. Sata *et al* (2000) report the onset of medial apoptosis within 2 hours of wire-insertion, and in this laboratory it has been previously demonstrated (also described in chapter 5) that this is followed by loss of medial cellularity and α-smooth muscle actin immunoreactivity for an extended period (at least 28 days; MacDonald *et al.*, Unpublished data). It is, therefore, likely

that the complete absence of vascular reactivity is a response to loss of those cells that normally mediate contractility. A second possibility is that wire-induced stretch has stripped any cells that survive from their connections to the surrounding cells/matrix, and as such, that contraction of individual cells is no longer coupled to contraction of the vessel. This suggestion is consistent with additional previous work in this lab in which contractility of wire-injured femoral arteries was also found to be abolished immediately after injury (MacDonald *et al.*, Unpublished data), possibly before substantial loss of medial cells could occur (Reis *et al.*, 2000; Sata *et al.*, 2000). Consideration must also be given to the neointima. Whilst it was not specifically shown in these vascular rings, it is assumed that as 14-day wire-injured vessels, they contain neointimal lesions of moderate extent (Sata *et al.*, 2000; Dover *et al.*, 2007). A large cellular mass residing within the vessel might physically prevent vascular reactivity, but this seems unlikely to be the sole explanation given the severity of the functional deficit. Also with regard to the neointima, it is interesting that these lesions, rich in smooth muscle-like cells are themselves incapable of mediating vasoconstriction. This probably reflects the synthetic phenotype typically adopted by initmal smooth muscle cells, in which, synthesis of contractile proteins is reduced (Ueda *et al.*, 1991; Leclerc *et al.*, 1992). Orientation of cells may also be important, as vasoconstriction probably requires a structured circumferential arrangement of contractile cells, whereas cells of the neointima are disordered, and even if capable of contraction, likely do so in opposition to one another.

Impaired contractility is also described in other intra-luminal models of acute vascular injury, although this is typically far less severe. Balloon-injured rat carotid arteries display reduced contractile response to phenylephrine (Heijenbrok *et al.*, 1998). Similarly, the same injury applied the rabbit carotid artery is said to result in reduced responsiveness to both phenylephrine and K^+ but not 5-HT or the thromboxane mimetic U46619 by Manderson *et al* (1989), suggesting modulation of specific agonist stimulation pathways by neointima formation. Azuma *et al* (1990), however, describe no such changes in contractility in the same model. Thus, some reduction in contractility may be common to models of intra-luminal vascular injury

with the severity probably determined by the severity of medial injury, which in the femoral artery wire-injury model is particularly great.

In models of this type, endothelium-dependent vasodilatation is also often reported to be impaired, even after EC regeneration. Reduced responsiveness to endothelium-dependent vasodilator agonists including acetylcholine, isoprenaline, 5-HT, the Ca^{2+} ionophore A23187 and ADP is variously reported in balloon-injured rabbit (Azuma *et al.*, 1990) and rat carotid (Heijenbrok *et al.*, 1998), rabbit iliac (Weidinger *et al.*, 1990) and porcine coronary arteries (Shimokawa *et al.*, 1987). This impairment is often, but not always agonist-dependent. Heijenbrok *et al* (1998), for example, report that vasodilator responsiveness to muscarinic receptor stimulation takes ~6 weeks to recover following balloon-injury to the rat carotid artery whilst α_2-adrenoceptor-mediated vasodilatation recovers within 2 weeks (Heijenbrok *et al.*, 2000). Further, similar dysfunction of responses mediated by re-generated EC are reported following *in vivo* denudation of the mouse carotid artery, an injury that does not induce neointima formation (Miller *et al.*, 2003). Thus, the endothelial dysfunction seen in these models may be a result of EC denudation and re-growth rather than a response to neointima formation. As it has not been possible to determine the extent of changes to endothelial function in the femoral artery wire-injury model, perhaps the most useful reports are those of EC function in the closely related mouse carotid wire-injury model. In this, EC-dependent vasodilatation to acetylcholine returns by day 10 and is only modestly impaired (~15%) indicating rapid recovery of EC which may, therefore, act to moderate lesion development (Liao *et al.*, 2007).

3.4.2 *Femoral artery ligation-injury impairs vascular function*

Although much less severe, altered vascular function was also detected in femoral arteries subject to either partial and complete ligation procedures; manifesting as impaired responsiveness both to acetylcholine and to vasoconstrictor substances. These defects may actually be an under-estimation of the functional consequences of ligation-injury because any changes are likely to be restricted to the vicinity of the ligature. Therefore, functionally normal portions of investigated vascular rings may have disguised more severe functional abnormalities present in injured sections.

These changes cannot be directly attributable to the presence of a neointima as, despite relatively similar functional impairments in partial and complete ligation models, partial ligation-injured vessels contained no more neointima than their sham-operated controls. For the same reason, the greater difficulty in mounting lesion-containing complete-ligation injured vessels and possible associated physical damage to EC or smooth muscle cells can be discounted.

In both partial and complete femoral artery ligation models, vasodilator responsiveness to acetylcholine, which in this artery probably reflects stimulation of NO production by EC (see chapter 5 and Crauwels *et al.*, 2000), was impaired (although the pattern of this defect was not identical). These changes are consistent with impaired acetylcholine-stimulated vasodilatation in rabbit carotid arteries injured by peri-vascular cuff placement, another non-denuding model of neointima formation (De Meyer *et al.*, 1991; De Meyer *et al.*, 1992). Many possible explanations have been proposed to mediate similar endothelial dysfunction in other situations of vascular injury, particularly in atherosclerosis, and may be applicable (reviewed by Harrison, 1997). Release of oxidative species from EC, smooth muscle cells or macrophages infiltrating the vascular wall or reduced production of anti-oxidant molecules, may result in increased oxidative deactivation of NO and thus impair its activity (Minor *et al.*, 1990). eNOS activity may also be reduced, either by reduced expression/phosphorylation (Wilcox *et al.*, 1997) or increased production of endogenous inhibitors. Balloon-injury to the rat carotid artery, for example, is associated with increased levels of the endogenous NOS inhibitor asymmetric-N,N-dimethylarginine, although this may simply be a response to the endothelial denudation and re-growth that occurs in the model (Azuma *et al.*, 1995b). Alternatively, the observed impairment of response to acetylcholine may reflect specific defects to muscarinic receptor signaling. Certainly, in the rabbit carotid artery peri-vascular cuff model, whilst the response to acetylcholine is impaired, vessels respond normally to other endothelium-dependent vasodilators including substance P, β-adrenoceptor agonists and Ca^{2+} ionophores (De Meyer *et al.*, 1991; De Meyer *et al.*, 1992). Further study would therefore be required to determine the exact nature of the mechanism for this defect. An attractive proposition for an

initiating stimulus for such changes to endothelial function in ligation-injured femoral arteries is alterations to arterial blood flow patterns that likely occur in both partial and complete ligation models (Sullivan & Hoying, 2002; Korshunov & Berk, 2003). Low or turbulent shear stress, detected through deformations of the EC cytoskeleton, is certainly known to modify the activity of many gene products critical to endothelial function. For example, low or oscillatory shear stress reduces expression and phosphorylation of eNOS (Corson *et al.*, 1996; Ziegler *et al.*, 1998; Jin *et al.*, 2003), its obligate co-factor tetrahydrobiopterin (Widder *et al.*, 2007) and the anti-oxidant enzyme Cu/Zn superoxide dismutase (Inoue *et al.*, 1996) whilst increasing expression of the superoxide generator NAD(P)H oxidase (De Keulenaer *et al.*, 1998).

Some further insight may be gleaned from the effect of sodium nitroprusside (SNP), a drug typically considered to induce vasodilatation by enzyme-catalysed release of NO from its structure. A small but significant impairment of the relaxant response to this agent was observed in complete but not in partial ligation-injured vessels. This reduced responsiveness implies that reduced bioactivity of NO, rather than altered production, may underlie the impaired response to acetylcholine. This may be because production of oxidative species is increased in complete ligation-injured vessels and thus, NO derived from both SNP and the endothelium is oxidatively deactivated before exerting a vasodilator effect. Further, NO release from SNP occurs intracellularly in smooth muscle cells, perhaps rendering it less sensitive to deactivation than endogenously produced NO which must diffuse further to exert its effect (Gruetter *et al.*, 1979). Therefore, the lesser effect of complete ligation on SNP-stimulated as compared to acetylcholine-stimulated vasodilatation may reflect the relative sites of NO release. An alternative possibility is that because responses to both exogenous and endogenous NO are impaired (although not to the same degree) that this defect reflects reduced sensitivity of the effector mechanisms of NO such as soluble guanylate cyclase activation.

Impaired absolute contractile responses to a variety of vasoconstrictor substances was also present in partial and complete ligation injured vessels. The apparent

agonist-independence of this defect suggests a reduction in inherent contractility rather than altered expression of particular receptor systems. No overt medial damage occurs in ligation models, making this contractile deficit more difficult to explain than that observed following wire-injury. Indeed, the presence of endothelial dysfunction might predict *increased* contractile responsiveness due to the loss of basal release of endothelium-derived relaxing factors (Waldron *et al.*, 1999; Crauwels *et al.*, 2000) as was observed in endothelium-denuded, un-injured femoral arteries. These data are, however, consistent with those from the rabbit carotid artery peri-vascular cuff placement model, in which (despite a similar absence of medial trauma) impaired contractile response to K^+, noradrenaline and U46619 were described (De Meyer *et al.*, 1990; De Meyer *et al.*, 1994). In contrast, 5-HT-stimulated contraction is enhanced by this injury (De Meyer *et al.*, 1990; De Meyer *et al.*, 1994), probably reflecting the increased expression of $5HT_{1B}$ receptors in this model (Geerts *et al.*, 2000). The mechanism behind this impairment of contraction is unclear. De Meyer *et al* (1994) suggest that this reflects an adoption of a synthetic phenotype by smooth muscle cells, which, therefore, exhibit reduced expression of contractile proteins. This is certainly plausible here given that flow-induced changes in endothelial function, tissue hypoxia, and/or macrophage infiltration into the medial layer may be present in both partial and complete ligation models and regulate smooth muscle phenotype (Thyberg, 1998). This suggestion is not consistent, however, with the maintained level of α-smooth muscle actin, a key contractile protein, in the media of ligation-injured vessels. Immunoreactivity for this antigen was even increased following complete ligation, which although suggestive of some change to smooth muscle phenotype might, again, be expected to result in *increased* rather than reduced responsiveness to vasoconstricting stimuli.

An alternative explanation is that impaired contractility in these ligation-injured vessels is a methodological artefact. It was not possible to determine optimal resting tension for injured vessels by normalisation as insufficient tissue was available for this procedure. As such, resting tension values determined previously for uninjured femoral arteries were utilised for both and sham and ligation-injured vessels. It may be, however, that application of excess passive tension caused smooth muscle cell

damage at the time of study. Similarly, as active contractility increases with the extent of stretch, reduced contractile responses of injured vessels could also result from application of sub-optimal resting tension (Mulvany & Halpern, 1977).

No change was noted in the responsiveness of vessels subject to partial or complete ligation to ET-1 as compared to their sham operated controls, beyond that which might be expected to result from altered basal contractility. It therefore appears that in this model neointima formation does not directly alter ET receptor function in cells mediating vasoconstriction: probably medial smooth muscle cells. It is curious, however, that in both complete ligation injured arteries and their sham operated controls, contractions induced by ET-1 were extremely weak, as compared to that seen in femoral arteries from un-operated mice. This may indicate a systemic alteration in ET receptor expression in this model, perhaps in response to the systemic inflammatory response (Pietersma *et al.*, 1995; Buffon *et al.*, 1999; Hojo *et al.*, 2001; Kochiadakis *et al.*, 2007) or elevation in circulating [ET-1] (Takase *et al.*, 2003) that is known to proceed acute vascular injury in man. More practical considerations, for example, contamination or low activity of the particular batch of ET-1 used in these tests cannot be excluded as no vessels from un-operated mice were studied in parallel.

In the case of both impaired endothelium-dependent vasodilatation and contractility a final question is why, in the partial ligation model, changes to vascular function were only present in the femoral artery proximal to the ligation and not the popliteal artery beyond it. This may simply be because ligation-induced changes to blood flow patterns do not extend distally and thus, that there is no stimulus for altered vascular function here. It may also reflect fundamental differences between these vessels. Certainly, vasomotor responses in popliteal arteries had several differences in characteristics compared to femoral arteries such as lesser contractile responsiveness, and an apparently bi-phasic rather than mono-phasic response to ET-1.

3.4.3 *Complete femoral artery ligation as a model of neointimal hyperplasia*

Complete ligation of the femoral artery resulted in formation of neointimal lesions in all vessels analysed. These lesions were broadly similar in composition to wire injury-induced lesions (see chapter 4 and 5) – rich in elastin, as indicated by United States trichrome staining, and smooth muscle-like cells, as indicated by α-smooth muscle actin immunohistochemistry. At the time point investigated, however, these lesions were ~50% smaller in absolute cross-sectional area than those in wire-injured arteries (chapter 4 and 5). Complete ligation-induced neointimal lesions were also a smaller than those reported to result from ligation of the carotid artery, by a similar degree (Kumar & Lindner, 1997). The former is easily attributable to the severity of the injury induced by wire-insertion. The latter may be a consequence of the larger calibre of the carotid than the femoral artery (Roque *et al.*, 2000; Kawasaki *et al.*, 2001). Indeed, the intima/media ratios observed here following complete femoral artery ligation are greater than those reported by Kumar *et al* (1997) in the carotid ligation model. Qualitative assessment of individual lesion cross-sections generated by OPT agree with histological analysis, and unsurprisingly indicate that wire-induced lesions afflict a greater length of vessel than do those induced by complete-ligation. Further, they confirm that in the partial ligation model, any morphological disturbances are restricted to the immediate vicinity of the ligature and, therefore, that the absence of lesions in myography rings from this model is not due to poor isolation and division of the injured artery.

The mechanism of lesion development in this model has not been investigated but several possible stimuli are present, including an inflammatory response to the ligature, local blood stasis, hypoxia and altered EC function (either reduced NO bioactivity or increased adhesion molecule expression). Some insight may be gleaned from the absence of neointima formation following partial femoral artery ligation. Thus, features that are common to both models may be eliminated from consideration as candidates for the primary stimulus of neointimal proliferation (although more subtle modulatory effects may still apply). In both models a ligature is present from the time of surgery, suggesting that either an inflammatory response to this, or its occlusion of the adventitial *vasa vasorum*, does not directly stimulate

neointimal hyperplasia. Lesion formation in response to severe hypoxic damage to vascular cells also seems unlikely as following ligation, the region is sufficiently nourished to support development of a large, richly cellular neointima.

Impaired endothelium-dependent vasodilatation is common to both models of arterial ligation. Endothelial dysfunction can certainly modulate neointima formation, as seen, for example, in eNOS$^{-/-}$ mice (Moroi *et al.*, 1998; Zhang *et al.*, 2006). Neither these animals, nor those in which the carotid artery endothelium is carefully denuded *in vivo*, spontaneously develop neointimal lesions however, indicating this too is not a primary cause of neointima formation (Miller *et al.*, 2003). Despite this, it cannot be excluded that impairment of stimulated NO release does not represent other changes in endothelial function. For example, adhesion molecule expression may be differentially regulated by complete and partial ligation. It would certainly be reasonable to expect that disturbances to blood flow are more substantial in the complete ligation model. If a modulatory role for the observed impairment of stimulated NO release at 28 days post-ligation is present, it may also be that changes in vascular function at this time point, do not reflect changes which are prevalent at earlier time points of active lesion growth.

This leaves blood stasis as a possible candidate for an initiating influence for neointimal proliferation. In the complete ligation model, some blood flow through arterial branches near to the femoropopliteal bifurcation may be preserved. Blood stasis, however, is likely to be much greater than following partial ligation, where any no-flow is probably restricted to a very small area immediately proximal to the ligature. Similarly, a lesser degree of blood stasis in a popliteal artery ligation model previously utilised in this laboratory (MacDonald *et al*, Unpublished data), may explain the comparatively small lesions resulting from this procedure. Moreover, studies of broadly analogous 'partial' ligations to the mouse carotid arteries are supportive of this dependence of lesion formation on the absence of flow. Ligation of the internal carotid just distal to the bifurcation of the superior thyroid artery results in 80% reduction in blood flow without appreciable neointima formation (Sullivan & Hoying, 2002). Similarly, ligation just distal to the occipital branch results in 90%

reduction of flow, with only minimal lesion formation (Korshunov & Berk, 2003). By comparison, the model of neointima formation described by Kumar & Lindner (1997) results from ligation just proximal to the occipital branch i.e. across the bifurcation of the common carotid artery, and therefore presumably results in complete cessation of flow.

Any regions of no-flow may allow the development of stasis thrombi, which secrete platelet-derived growth factor and other mitogens and provide a framework into which smooth muscle cells can migrate to form a neointima - a mechanism of lesion formation more reminiscent of that suggested by Schwartz *et al* (1992a) than the conventional model (Libby *et al.*, 1992). Certainly, in the mouse carotid artery ligation model, thrombi are described to occur in the region of injury (Kumar *et al.*, 1997; Kumar & Lindner, 1997; Kawasaki *et al.*, 2001). Whilst none have been observed in these studies of femoral artery ligation, any thrombotic response is likely to have resolved by the 28 day post-injury time-point investigated. Further, the carotid artery ligation model is sensitive to manipulations to processes of coagulation, fibrinolysis and platelet aggregation (Kawasaki *et al.*, 2001; Wang *et al.*, 2005b; Martin-McNulty *et al.*, 2007)- supporting the view that thrombus formation is important in ligation-induced lesion formation. Therefore, whilst somewhat speculative, it is tempting to suggest that the degree of neointimal lesion formation resulting from ligations about the femoropopliteal bifurcation (as for the carotid) is a response to the degree of blood stasis and thrombus formation resulting from each procedure.

3.4.4 *Optical projection tomography for the analysis of neointima formation*

Data from the three-dimensional OPT imaging of injured femoral arteries indicate that this technique offers potential advantages in the measurement of vascular lesion formation in small arteries. The qualitative similarities between images produced by this method, and by subsequent histological analysis of the same vessels, are striking and this is confirmed by strong correlations between planimetric measurements of neointimal and luminal cross-sectional areas made between these techniques. Any deviations in these correlations are likely to be a consequence of the difficulties in

identifying the exact same regions in histological sections and OPT scans and, as such, may reflect a limitation of the comparison method rather than the imaging technique. Alternatively, because the delineation of the neointima/media border is, in some cases, subjective, inclusion of portions of media in neointimal measurements may provide an additional source of variability. If this were common, however, a positive bias in the correlation between OPT and histology measures of neointima might be expected, and none was apparent. Regardless, the greater power of this technique is in the assessment of volumetric parameters and these are likely to be relatively insensitive to small, unbiased errors in individual 2D measurements.

OPT offers a number of potential benefits over traditional histological methods of lesion analysis. Analysis of long lengths of vessels by microtomy of serial sections and histological staining is both extremely time-consuming and labour-intensive. OPT analysis in comparison is rapid, requires less user input and, after scanning, tissues remain undamaged for further use (including histological analysis). Also for reasons of practicallity, histological analysis is typically limited to description of injured vessels by one site in which the largest lesions are present. Full 3D analysis of lesion size and distribution, however, may offer greater power to detect an effect of intervention, and describe more subtle changes in lesion development (McAteer *et al.*, 2004). Given the unproven nature of this technique, the workflow adopted for this thesis was of OPT scanning before full histological analysis. In future, a better compromise between these potentially complementary methods might be adopted - to determine lesion volume and distribution by OPT, and to selectively analyse only the regions containing the largest lesions in detail using histological means.

Surprisingly few alternative methods for the 3D imaging of small vascular lesions have been described, perhaps reflecting a greater interest in *in vivo* imaging modalities. One relevant outcome of this focus is the possible use of MRI for the analysis of tissues *ex vivo,* which allows for greater resolution than is possible *in vivo.* McAteer *et al* (2004) have described the use of this technique for the analysis of atherosclerotic lesions in the brachiocephalic artery, but the work performed in this chapter indicates OPT may be equivalent or superior. Even though the resolution

of these *ex vivo* MRI images appears similar to those produced by OPT, it must be considered that this only because the brachiocephalic is substantially larger than the femoral artery. Of more practical concern, image acquisition time to produce such MRI images is 7 hours per vessel, despite use of an exceptionally powerful scanner (11.7 Tesla). For comparison, OPT images are acquired in <20 mins using a tomograph of only modest expense. OPT also possesses the potential to visualise the extent and distribution of particular antigens. Whole mount immunoflourescent staining of tissue samples can be imaged alongside basic morphology (i.e. tissue auto-fluorescence) using an alternative UV filter set. Whilst an exciting possibility, attempts to utilise this process here have been frustrated by difficulties associated with antibody penetration and removal from intact tissues. Other possible techniques for the 3D imaging of blood vessels such as optical coherence tomography and whole-mount confocal microscopy remain to be investigated. In the latter case, whilst this method might offer superior resolution to all others discussed, any use may be severely limited by depth of sample that can be studied.

3.4.5 *Conclusions*

The studies presented in this chapter describe the functional and morphological consequences of a novel model of femoral artery ligation-induced neointimal hyperplasia, and a new technique for rapidly analyzing vascular lesion formation in three dimensions. The complete femoral artery ligation model will provide a useful complement to the wire-injury model owing to the absence of physical damage to the endothelium. This is of particular importance given the hypothesis that ETB expressed by EC modulates the response to vascular injury, and that it has not been possible to determine how well endothelial function returns following wire-injury. Finally, OPT appears to be well suited to the 3D imaging of injured femoral arteries and should allow the assessment of volumetric parameters and lesion distribution following injury, and how these parameters respond to interventions.

Chapter 4

Effects of systemic pharmacological ET receptor blockade on neointimal lesion formation

4.1 INTRODUCTION

There is little doubt that acute injury to the vascular wall provokes activation of the endothelin (ET) system, for example, resulting in both local (Kurata *et al.*, 1995; Dashwood *et al.*, 1999; Shirai *et al.*, 2006) and systemic (Takase *et al.*, 2003) increases in the tissue and circulating levels of the ET peptides in human patients. Interventional studies in animals have established that endogenous ETs are not simply a marker of disease progression but are themselves powerful stimulants for the development of neointimal hyperplasia. This literature consistently states that selective blockade of the ETA receptor is sufficient to moderate neointimal thickening following various insults to the vascular wall, in a variety of non-human species (Ferrer *et al.*, 1995; Takiguchi & Sogabe, 1996; Burke *et al.*, 1997; McKenna *et al.*, 1998; Dashwood *et al.*, 1999; Murakoshi *et al.*, 2002; Tepe *et al.*, 2002; Wan *et al.*, 2004).

The role of the ETB receptor in vascular lesion formation following injury is more difficult to deduce. Certainly, ETB receptors expressed by vascular smooth muscle cells (VSMC) and leucocytes may mediate many actions common to ETA; such as promotion of mitogenesis (Eguchi *et al.*, 1994; Yang *et al.*, 1999; Di Luozzo *et al.*, 2000), monocyte chemotaxis (Achmad & Rao, 1992; Cui *et al.*, 2001; Grimshaw *et al.*, 2002) and cytokine production (Ruetten & Thiemermann, 1997; Shimada *et al.*, 1998; Speciale *et al.*, 1998; Juergens *et al.*, 2008). On endothelial cells (EC) and collecting duct epithelial cells (IMCD), however, the ETB receptor may mediate actions that might plausibly limit the response to vascular injury such as induction of nitric oxide (NO) release by EC, clearance of circulating ET-1 and natriuresis (Bagnall *et al.*, 2006; Ge *et al.*, 2006). Therefore, whilst activation of ETA and ETB on VSMCs and leucocytes probably contributes to neointima formation, this may be moderated by local (NO release) and systemic actions (ET-1 clearance, natriuresis) mediated by the ETB receptor.

The body of work that describes the effects of ETB blockade in neointimal hyperplasia is both small and inconsistent. On one hand, most (Douglas *et al.*, 1994; Ohlstein *et al.*, 1994; Douglas *et al.*, 1995a; Tsujino *et al.*, 1995; Chandra *et al.*,

1998; Marano *et al.*, 1998; Azuma *et al.*, 1999; Sanmartin *et al.*, 2003; Reel *et al.*, 2005) but not all (Huckle *et al.*, 2001) studies that investigate the effects of non-selective ETA/ETB blockade on neointima formation demonstrate a reduction in lesion size. This might suggest that any contribution of ETB is modest since ETA blockade alone has a similar effect but no direct comparisons of the magnitude of these changes have been reported. On the other hand, Murakoshi *et al* (2002) report that lesions resulting from carotid artery ligation are increased in size, both in "rescued" ETB knockout mice and in wild-type mice treated with a selective ETB antagonist. This is associated with reduced tissue levels of NO oxidation products suggesting that NO release from EC may be important, particularly because this model does not feature physical damage to endothelium.

The work in this chapter addressed the hypothesis that endogenous ET-1 stimulates neointimal proliferation after arterial injury by activation of the ETA receptor subtype but that this action is opposed by concurrent ETB receptor stimulation. To examine this, the effects of selective pharmacological ETA and ETB receptor blockade, both alone and in combination, were observed. The possible requirement of a physically-intact endothelium on the role of both receptors was assessed by use of both wire-induced (endothelium-denuding) and ligation-induced (endothelium-intact) models of vascular injury.

4.2 METHODS

4.2.1 *Animals*

Experiments were carried out on male 25-30g C57Bl/6J mice purchased from Harlan Laboratories, UK. Animals were ear-clipped for identification purposes and allowed to acclimate to their environment for at least one week before further use.

4.2.2 *Drug administration*

The orally active endothelin receptor antagonists atrasentan (2000-fold ETA-selective; $10mg.kg^{-1}.day^{-1}$), A192621 (1300-fold ETB-selective; $30mg.kg^{-1}.day^{-1}$) individually or in combination were administered in chow. These doses have been shown previously to achieve strong and selective blockade of their respective targets in rodents, as assessed by alterations to the ETB-mediated depressor and ETA-mediated pressor phases of the response to acute ET-1 administration (Opgenorth *et al.*, 1996; Wessale *et al.*, 2002). Drug solutions, prepared by dispersion of the relevant agent(s) in 0.2% methylcellulose were bound with RM1 mouse chow with beef gelatin as described in section 2.3.2. Drugged diet was packed into 60ml centrifuge tubes for consumption, providing at least 1 tube for every 2 mice. Diet was checked daily for depletion or signs of spoilage and replaced as necessary. Tubes were regularly weighed to monitor consumption. Drug diet was administered from 1 week prior to femoral artery injury surgery until the completion of the study (**Figure 4-1**).

4.2.3 *Blood pressure*

To verify effective absorption and activity of ET receptor antagonists systolic blood pressure was measured over the study period using the technique of tail cuff plethysmography (section 2.3.3). To familiarise animals to handling, restraint and cuff inflation, blood pressure measurements were commenced 2 weeks prior to the period of drug administration, and were recorded twice-weekly until femoral artery injury surgery was performed. Following a 10 day post-operative recovery period, systolic blood pressure measurements were performed weekly until study completion

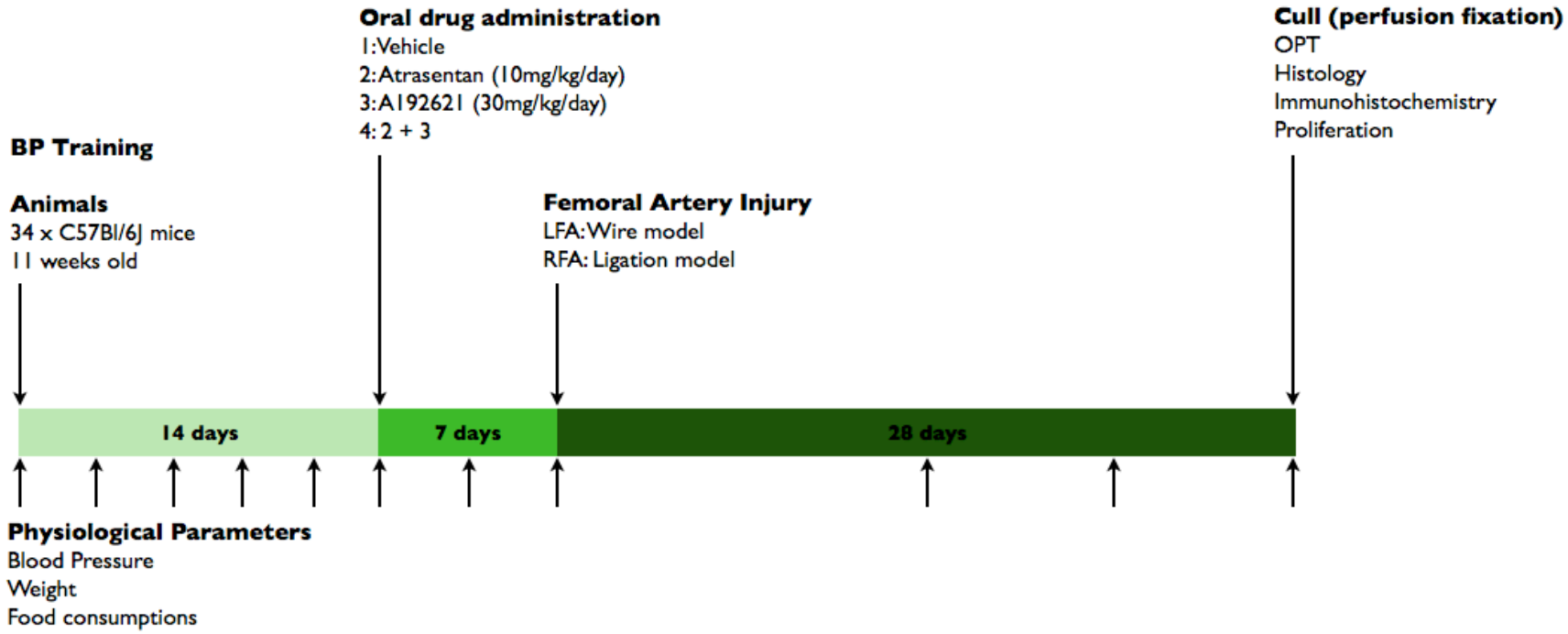


Figure 4-1 Study protocol to determine the effects of ET receptor blockade on neointima formation.

Experiments were conducted in male, 11 week old C57Bl6J mice. Blood pressure was measured twice weekly by tail cuff plethysmography during a 21 day run-in period. On day 14, animals were placed on diet containing 1: vehicle (n=10), 2: atrasentan (10mg/kg/day; n=8), 3: A192621 (30mg/kg/day; n=8) or 4: atrasentan + A192621 (10mg/kg/day and 30mg/kg/day, respectively; n=8) and remained so for the study duration. At the end of this period (day 21), each mouse underwent left femoral artery wire-injury and right femoral artery complete ligation-injury. Animals were allowed to recover for a further 28 days, during which, after a 10 day rest period, blood pressure was measured weekly. At the end of this (day 49), mice were culled by perfusion fixation and injured femoral arteries isolated for examination by OPT, histology and immunohistochemistry. Food consumption and body weight were recorded at the same intervals as blood pressure measurement, throughout the study. LFA, left femoral artery; RFA, right femoral artery; OPT, optical projection tomography.

(**Figure 4-1**). Animals were weighed and body weights recorded according to the same schedule.

4.2.4 *Femoral artery injury*

To induce neointimal hyperplasia, two models of femoral artery injury were employed as detailed in section 2.3.1. Both procedures were performed in each mouse to minimise the number of animals and amount of drugged diet required (**Figure 4-1**). Previous studies in our laboratory have demonstrated that dramatic alterations in lesion size (produced by local drug delivery) have no effect on the neointimal thickening in the contra-lateral artery, suggesting that that there is little potential for interaction between wire- and ligation- injuries performed in the same animals (MacDonald *et al.*, Unpublished data). Further, multiple models of neointimal proliferation in a single mouse have been used previously to assess the mechanisms of remodelling (Tanaka *et al.*, 2003).

Intra-luminal wire-injury was inflicted to the left femoral artery of each mouse, using the method of Sata *et al* (2000), wherein a 0.014" diameter straight sprung angioplasty guide wire is advanced ~1.5cm proximally into the isolated femoral artery through an arteriotomy in the popliteal branch. In the contra-lateral (right) femoral artery, injury was induced using an adaptation of the model of carotid artery ligation (Kumar & Lindner, 1997). The isolated femoral artery was ligated with 5-0 silk at its junction with the popliteal artery. Mice were allowed to recover for 28 days to permit lesion development in both arteries after which mice were killed by exsanguination and trans-cardiac perfusion fixation under terminal pentobarbital anesthesia. Two hours prior to induction of terminal anaesthesia, 5-bromo-2-deoxyuridine ($100mg.kg^{-1}$, i.p.) was administered to allow assessment of cell proliferation. Vessels were isolated, cleaned of extraneous peri-adventitial material and their identity blinded before subsequent analysis.

4.2.5 *Optical projection tomography (OPT)*

For three-dimensional evaluation of injured femoral arteries, vessels were embedded in agarose for analysis by OPT (section 2.5). Briefly, after dehydration in methanol and refractive index matching in benzyl alcohol/benzyl benzoate, each for 24 hours, vessels were scanned for fluorescent emission (excitation filter: 425/40nM; emission filter: 475nm low pass) using a Bioptonics OPT tomograph with a scanning resolution of 1.048Mpixels. Computed tomography reconstruction was performed by filtered back-projection of raw data using NRecon software. Volumetric measurements were acquired by manual tracing of the estimated position of the internal elastic lamina and subsequent grey-level thresholding to delineate neointima from lumen.

4.2.6 *Histology*

Following acquisition of OPT scans, vessels were prepared for histological evaluation by washing in methanol (>24 hours) before processing to paraffin wax. To reduce the required amount of microtomy, several arteries were embedded in each paraffin block. 3µm serial sections were cut at intervals of 100µm and 50µm for wire- and ligation-injured femoral arteries, respectively. At each interval, 1 section was stained using the 'United States trichrome' method (Hadoke *et al.*, 1995) to highlight the borders of each arterial layer, and to locate the region bearing the largest neointimal lesion. All further histological/compositional analysis was carried out on sections adjacent to this region. For ligation-injured arteries, only vessels containing patent lumen were considered for analysis to exclude areas crushed by the application of the ligature. Measurements of intimal area, medial area and luminal area were recorded using image analysis software (Photoshop CS3 Extended, Abode Systems, USA) and intima/media ratio and stenotic ratios calculated. For evaluation of lesion collagen content, adjacent sections were stained with picro-sirius red and the area of staining within the neointimal layer calculated by colour deconvolution and expressed as a percentage of total neointimal area using Photoshop CS3 Extended software (Abode Systems, USA).

4.2.7 *Immunohistochemistry*

For analysis of lesion composition and cell proliferation, immunohistochemistry was performed on sections from each vessel adjacent to the region identified as having the largest neointimal lesion. Reactions were performed according to the immunoperoxidase, labelled streptavidin-biotin method using primary antibodies reactive to α-smooth muscle actin (αSMA), Mac2 and bromodeoxyuridine (BrdU). αSMA and Mac2 immunoreactivity were quantified by semi-automated colour deconvolution (Photoshop CS3 Extended, Abode Systems, USA) and expressed as a fraction of the total area of the arterial layer (media, neointima) of interest. BrdU incorporation was recorded by blinded, manual count of immunoreactive nuclei.

4.2.8 *Statistics*

All statistical analysis was performed by one-way ANOVA with Dunnet's post-hoc test for comparison of drug treatments with vehicle, using Prism 4.0 software (Graphpad software, USA). Systolic blood pressure for each animal was taken as the mean of the measurement taken immediately prior to induction of vascular injury, and the 3 measurements taken over the recovery period.

4.3 RESULTS

4.3.1 *Physiological parameters*

Prior to administration of pharmacological agents, systolic blood pressure (SBP), as measured by tail cuff plethysmography, was of expected physiological value and not significantly different between intended treatment groups (vehicle: 101.4±1.2mmHg; atrasentan: 100.0±1.4mmHg; A192621: 98.6±0.9mmHg; atrasentan+A192621: 100.7±1.3mmHg; p=0.434). Similarly, pre-study body weight was not different between groups (**Table 4-1**, page 146). During the period of drug dosing, SBP (**Figure 4-2**) was decreased by 6.5mmHg and 6.3mmHg, respectively, for atrasentan- and atrasentan+A192621-treated animals (both $p<0.01$), and increased by 8.3 mmHg in mice treated with A192621 alone ($p<0.001$). The rate of change of body weight was not significantly altered by treatments (**Table 4-1**, page 146). Consumption of drugged food was consistent with predicted intake of $10g.day^{-1}.mouse^{-1}$ over the period of drug dosing (vehicle: $10.3g.day^{-1}.mouse^{-1}$; atrasentan: $9.4g.day^{-1}.mouse^{-1}$; A192621: $9.4g.day^{-1}.mouse^{-1}$; atrasentan+A192621: $10.3g.day^{-1}.mouse^{-1}$). A moderate drop (<50%) in consumption of diet was noted in the immediate post-operative period but returned to normal levels within 3 days. At the study end, obvious evidence of infection was noted at the surgical site in several mice (1 vehicle-treated, 1 atrasentan-treated, 1 A192621-treated). Vessels from these animals were excluded from further analysis.

4.3.2 *Wire injury*

4.3.2.1 *3D morphometry – wire injury*

Optical projection tomography was used to evaluate the total lesion and luminal volumes of wire-injured femoral arteries. As in chapter 3, neointimal lesions were clearly visible in reconstructed OPT scans and were present in all treatment groups. Treatment of animals with A192621 (10mg.kg-1.day-1) was associated with a significant increase in the fraction of the total vessel volume occupied by wire-induced lesion ('volumetric stenotic ratio'; **Figure 4-3**). Trends towards reduced luminal volume ($p>0.05$) and increased neointima volume ($p>0.05$) were also

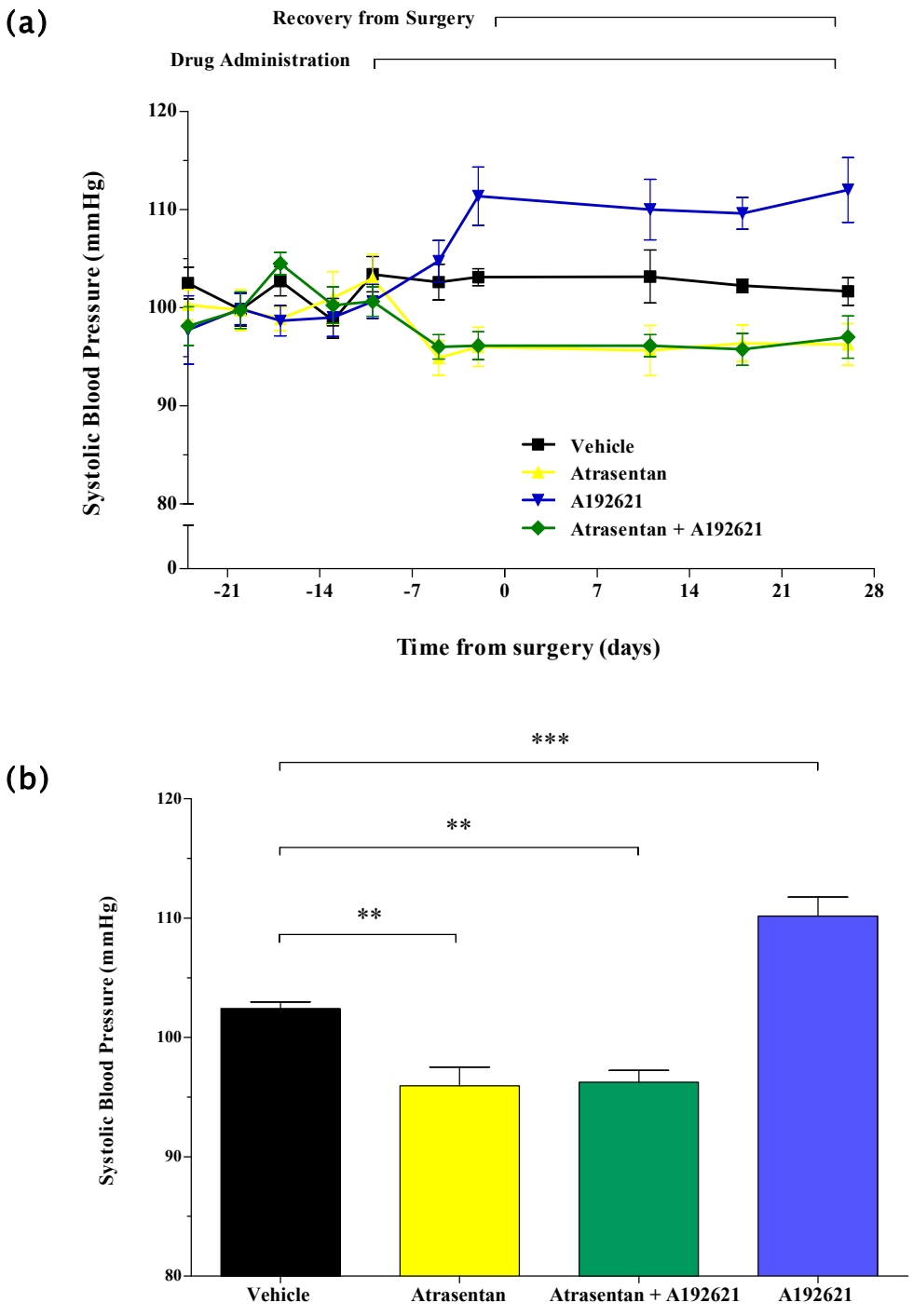


Figure 4-2 Impact of endothelin receptor blockade on systolic blood pressure.

SBP was measured in mice subject to femoral artery injury and treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) using tail cuff plethysmography. Pre-drug administration SBPs were similar between all groups (a). SBP was reduced by ETA and ETA+ETB blockade, and increased by ETB blockade and these effects were maintained over the treatment period (a). As such, mean SBPs over the study duration were significantly increased by A192621 and decreased by atrasentan ± A192621 (b). n=8-10. Data are mean ± SEM. SBP, systolic blood pressure; **, $p<0.01$; ***, $p<0.001$ by two-way ANOVA with Dunnet's post-test.

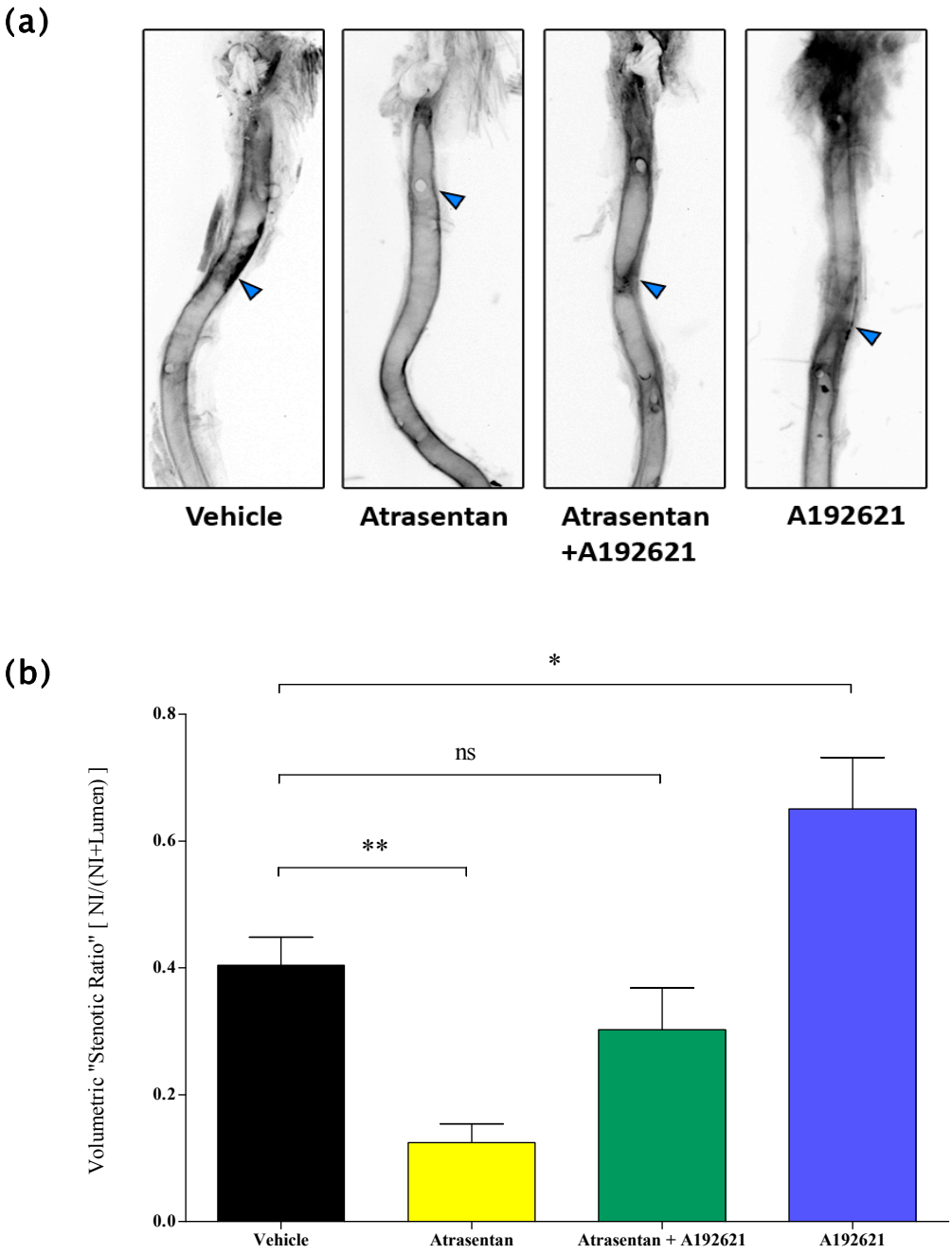


Figure 4-3 Impact of endothelin receptor blockade on wire injury-induced lesion volume, as assessed by optical projection tomography.

Neointima formation was induced by femoral artery wire-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before isolation and optical projection tomographic scanning. Lesions were clearly visible in raw projection images (a, arrowheads). Lesion volumes were recorded from reconstructed tomograms and expressed as a fraction of vessel occupied by lesion. This measure was significantly reduced by ETA blockade, increased by ETB blockade and not altered by ETA+ETB blockade. n=6-8. Data are mean ± SEM. ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$ by two-way ANOVA with Dunnet's post-test.

apparent in this treatment group, but neither reached statistical significance (**Table 4-2**, page 147). In animals treated with atrasentan (30mg.kg-1.day-1), the fraction of vessel volume occupied by lesion was significantly reduced by treatment (**Figure 4-3**), but again, trends toward reduced absolute neointimal volume, and increased luminal volume did not attain statistical significance (**Table 4-2**, page 147). No changes in any measure of 3D lesion morphology were apparent in mice treated with the combination of atrasentan and A192621 – volumetric stenotic ratio (**Figure 4-3**) and neointimal volume were similar to those in arteries from vehicle treated mice, and a trend towards increased luminal volume was not significant (**Table 4-2**, page 147).

4.3.2.2 *2D morphometry – wire injury*

Histological analysis of the largest wire-induced lesions in each vessel provided confirmatory data. Femoral artery wire injury resulted in formation of fibro-proliferative neointimal lesions in all treatment groups (**Figure 4-4**). Selective ETB blockade with A192621 was associated with increased maximum lesion size (**Figure 4-5**) and intima/media ratio (**Table 4-2**, page 147). No changes in luminal area, medial area or the stenotic ratio were apparent in arteries from this group. As such, A192621 was associated with an increase in the total area inside the external elastic lamina (**Table 4-2**, page 147).

In animals treated with the selective ETA antagonist, atrasentan, an increase in luminal area at the point of maximum neointimal lesion size was observed in wire-injured femoral arteries (**Figure 4-5**), and accordingly, stenotic ratio was significantly decreased (**Table 4-2**, page 147). A trend towards reduce neointimal lesion size and intima/media ratio was also apparent in this group, but did not reach significance ($p>0.05$; **Figure 4-5**). Medial area was also unaltered by atrasentan treatment. As such, outward structural dilation would appear to contribute to the increased luminal area, although the total area inside the external elastic lamina was not significantly increased (**Table 4-2**, page 147).

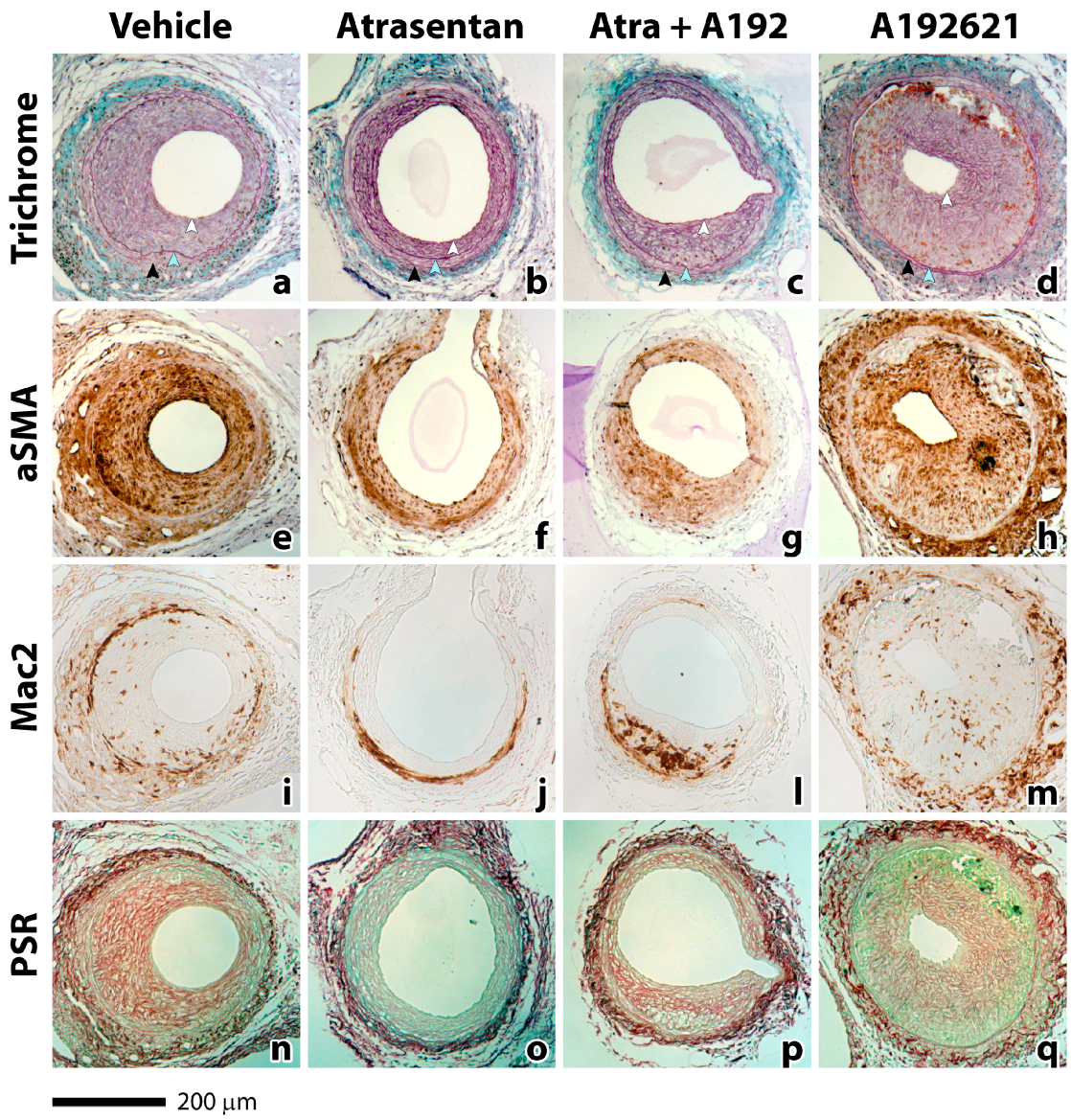


Figure 4-4 Impact of endothelin receptor blockade on femoral artery wire injury-induced lesion size and composition.

Neointima formation was induced by femoral artery wire-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before histological examination. Representative photomicrographs of lesions from all treatment groups stained by United States Trichrome (a-d), Picro-sirius red (n-q) and immunohistochemistries for αSMA (e-h) and Mac2 (i-m), are shown. Trichrome staining reveals formation of large, elastin (purple) and collagen (blue)-rich lesions in all groups which are reduced and increased in size by ETA (b) and ETB blockade (d), respectively (a-d). From these, the lumen/intima (white arrowheads), intima/media (blue arrowheads) and media/adventitia border (black arrowheads) can be clearly seen. All lesions are rich in immunoreactivity for αSMA which is also present in the media, and often, adventitia (e-h). Mac2 immunoreactivity is also found throughout the vascular wall in injured vessels, although the extent is much less. Picro-sirius red staining further reveals the high collagen content (red) of these lesions (n-q) which is somewhat lower in those from mice treated with ETA (o) or ETA+ETB blockade (p). αSMA, α-smooth muscle actin; PSR, picro-sirius red.

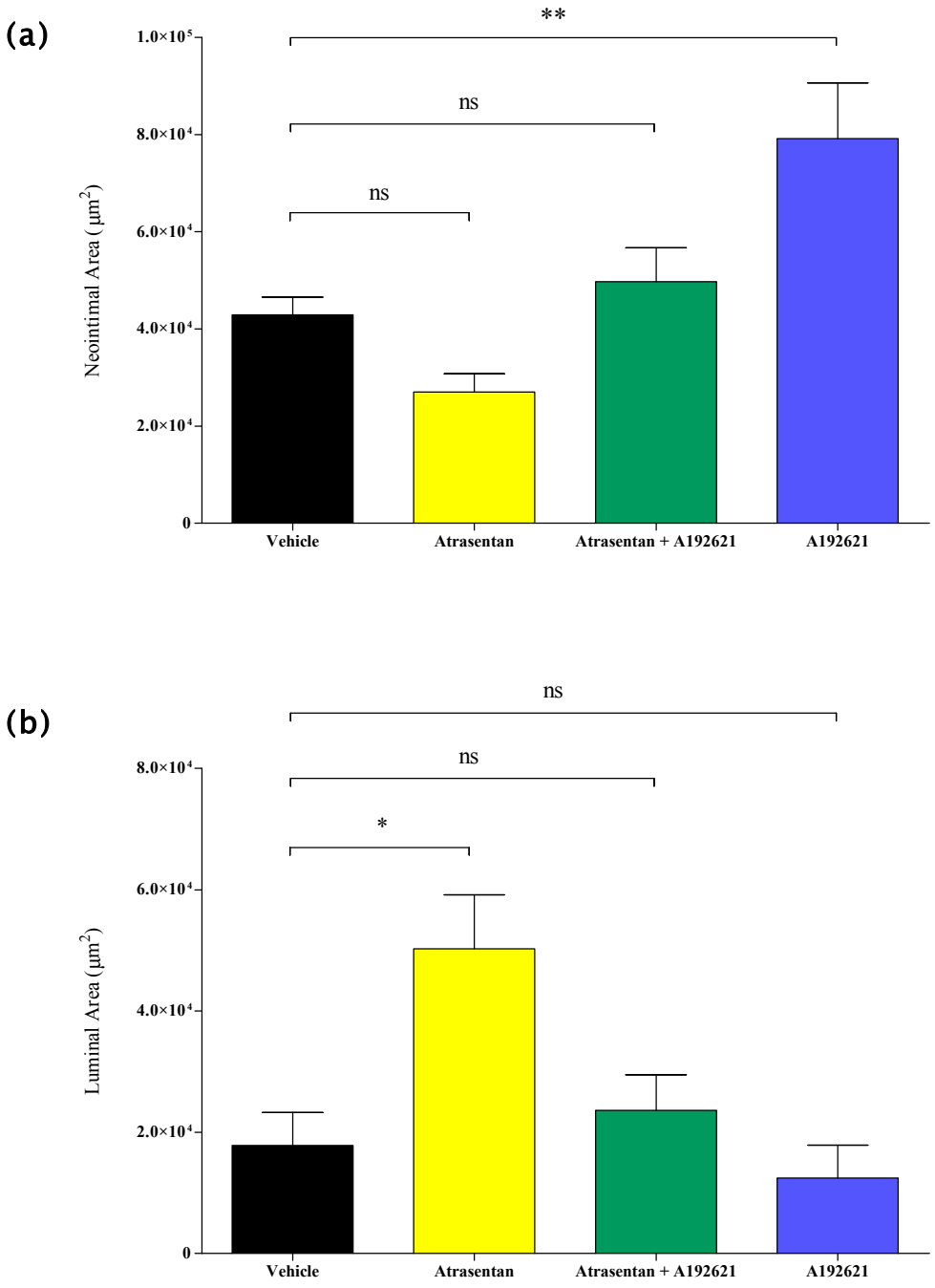


Figure 4-5 Impact of endothelin receptor blockade on neointimal and lumenal area in wire-injured femoral arteries, as assessed by histology.

Neointima formation was induced by femoral artery wire-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before histological examination. Neointimal (a) and lumenal cross-sectional areas (b) were measured at the point of largest lesion size by morphometry. Neointimal area was increased in mice subject to ETB blockade (a). A trend towards reduced lesion size ($p>0.05$) was apparent with ETA blockade. ETA blockade did significantly increase lumenal area (b). Lumenal cross-sectional was not altered by ETB or ETA+ETB blockade. n=7-9. Data are mean ± SEM. ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$ by two-way ANOVA with Dunnet's post-test.

Combined blockade of ETA and ETB receptors by treatment with both atrasentan and A192621 was not associated with any change in histological measures of luminal, medial or neointimal area (**Figure 4-5**). Consistently, the total area inside the external elastic lamina was similar to that in vessels from vehicle treated animals (**Table 4-2**, page 147).

4.3.2.3 *Lesion composition – wire injury*

Immunoreactivity for α-smooth muscle actin, indicating VSMC and myofibroblast content, was detected throughout the neointima and media in all treatment groups (**Figure 4-4**). The fractional area of immunoreactivity was not different between treatment groups in either layer of wire-injured femoral arteries (**Table 4-2**, page 147).

Mac2 immunoreactivity, a marker of macrophage content, was typically present only at a low level, but was variably associated with the adventitia, media and neointima (**Figure 4-4**). The fraction of neointimal and medial area immunoreactive for Mac2 was also not significantly different between treatment groups. A non-significant trend towards increased fractional Mac2 immunoreactive area, however, was noted in the neointima of A192621 treated animals, with or without concurrent ETA receptor blockade (both $p>0.05$; **Figure 4-6**).

Lesion collagen content was evaluated by histological staining with picro-sirius red, and was abundant in all layers of injured vascular wall (**Figure 4-4**). The fraction of the neointima staining for collagen was significantly lower in injured femoral arteries from ETA/ETB receptor antagonist-treated animals (**Figure 4-6**). A non-significant trend towards decreased collagen area was also apparent in lesions from mice treated only with an ETA antagonist. In wire injury-induced neointimal lesions from ETB antagonist treated mice, lesion collagen content was similar to that in those from vehicle-treated animals.

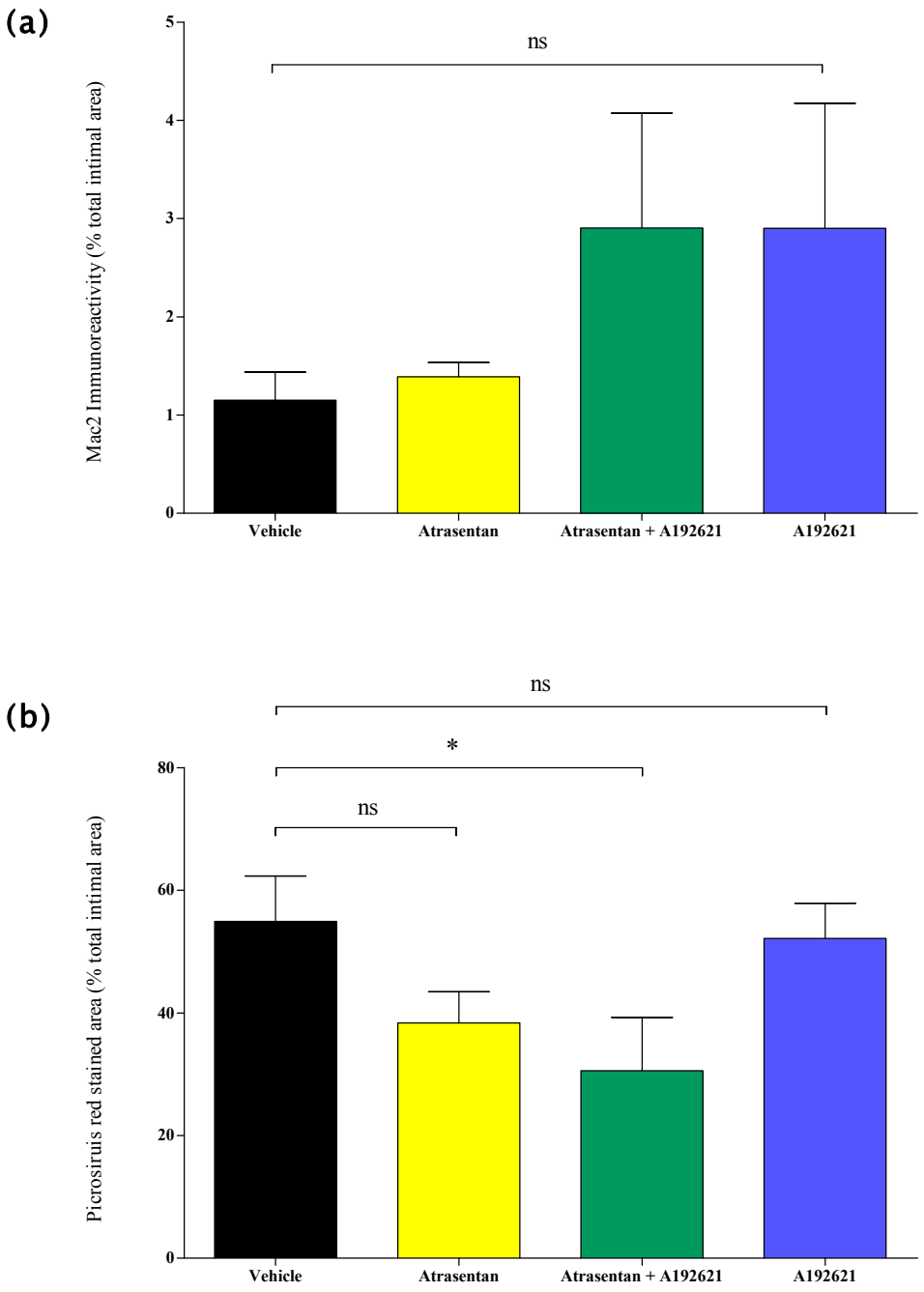


Figure 4-6 Impact of endothelin receptor blockade on macrophage and collagen content of wire injury-induced neointimal lesions.

Neointima formation was induced by femoral artery wire-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before histological examination. Macrophage content was identified by Mac2 immunoreactivity and collagen content picro-sirius red staining in the largest lesions from each vessel. Results were expressed as % of neointimal area stained. A trend ($p>0.05$) towards increased neointimal Mac2 content was present in mice treated with ETB or ETA+ETB antagonists (a). The combination of ETA+ETB blockade significantly reduced neointimal collagen content (b). A trend ($p>0.05$) towards reduced collagen content was also seen with ETA blockade. n=7-9. Data are mean ± SEM. ns, $p>0.05$; *, $p<0.05$; by two-way ANOVA with Dunnet's post-test.

Measurement of BrdU incorporation indicated few cells in S phase at this point of lesion development in either the media or neointima of wire-injured vessels from mice treated with vehicle, atrasentan or the combination of atrasentan and A192621 (**Table 4-2**, page 147). In the intimal lesions of mice treated with A192621 alone, however, a significant increase in the number of proliferating cells was observed.

4.3.3 *Ligation injury*

4.3.3.1 *3D morphometry – ligation injury*

The presence and distribution of neointimal lesions in femoral arteries subject to complete ligation was clearly indicated by OPT reconstructions, as described in chapter 3. In the typical ligation-injured vessel, immediately proximal to the ligation, an occluding, cellular neointima was present. Beyond this, the lesion was seen to gradually dwindle to a morphologically normal artery. In this area of transition, a variably-sized lesion was present surrounding patent lumen, typically connecting with an arterial branch. Although in most vessels, no further disturbances were observed proximal to this, in a small fraction, intermittent regions of intimal hyperplasia were seen (**Figure 4-7**).

In mice treated with the ETB antagonist A192621, no changes in 3D morphological parameters were apparent – luminal volume, neointimal volume and volumetric stenotic ratio (**Figure 4-7**) were similar to those in arteries from vehicle treated mice (**Table 4-3**, page 148). Selective ETA blockade by atrasentan treatment, however, was associated with a reduction in the fraction of vessel volume occupied by neointimal lesion (**Figure 4-7**), and trends (both $p>0.05$) towards reduced neointimal volume and increased luminal volume (**Table 4-3**, page 148). Similar changes were present in mice treated with the combination of ETA and ETB antagonists, with a significant reduction of volumetric stenotic ratio (**Figure 4-7**) and non-significant trends towards increased luminal volume and decreased neointimal volume (**Table 4-3**, page 148).

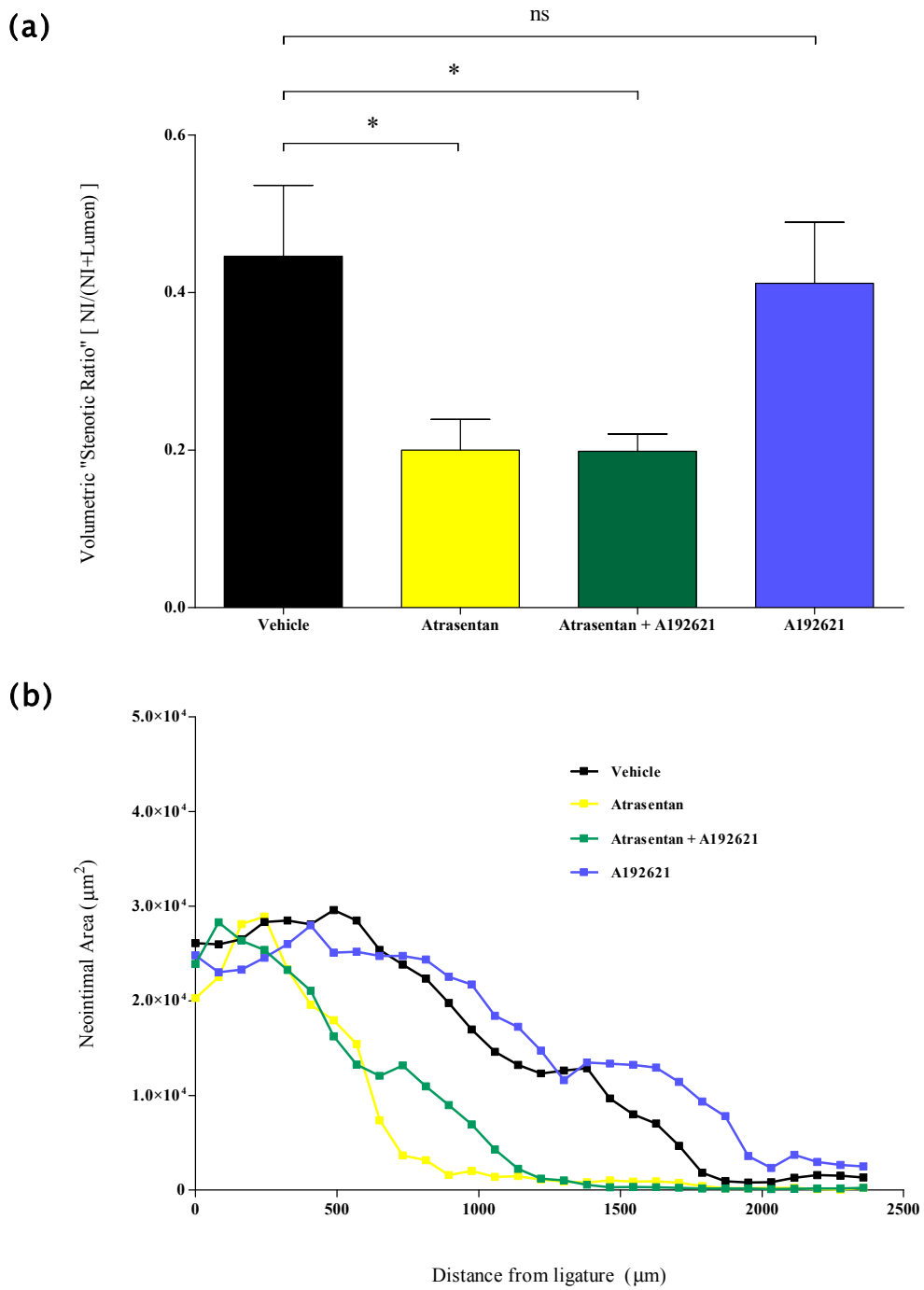


Figure 4-7 Impact of endothelin receptor blockade on neointimal volume and axial distribution in complete ligation-injured femoral arteries, as assessed by optical projection tomography.

Neointima formation was induced by complete femoral artery ligation-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before optical projection tomographic evaluation. From reconstructed tomograms, neointima was delineated and expressed as a fraction of vessel occupied by lesion (a) and by plotting cross-sectional area against distance from the ligature (b). Neointimal volume fraction was significantly reduced by ETA and ETA+ETB blockade but not altered by ETB blockade (a). Axial profiles of lesion size indicated that this effect was mediated reduction of the axial lesion extent, rather than maximum lesion size (b). n=5-7. Data in (a) are mean ± SEM. Data in (b) are mean only, SEM is omitted here for clarity. ns, $p>0.05$ by two-way ANOVA with Dunnet's post-test.

Longitudinal profiles of lesion size along the length of ligation-injured vessels were produced from OPT scans to describe the distribution of lesion (**Figure 4-7**). These indicated that any reduction in lesion size associated with ETA blockade (with or without concurrent ETB blockade) was due to a decrease in the axial length of lesions rather than in maximum lesion cross-sectional area.

4.3.3.2 *2D morphometry – ligation injury*

Analysis of ligation-induced lesion formation in the femoral artery by histology also demonstrated the presence of fibro-proliferative neointima formation, although these were somewhat smaller than those found in their wire-injured counterparts (**Figure 4-8**). In contrast to volumetric data by OPT, no significant changes in any histological measures including the areas of the largest neointimal lesions (**Figure 4-9**), the lumen and the media, was associated with any treatment group (**Table 4-3**, page 148). This is consistent with the OPT generated lesion profiles, which indicate that lesion length rather than maximum lesion size is modulated by ETA blockade (**Figure 4-7**).

4.3.3.3 *Lesion composition – ligation injury*

As in wire-injured vessels, immunoreactivity for α-smooth muscle actin immunoreactivity was abundant throughout the media and neointima of ligation-injured arteries (**Figure 4-8**). The fractional area of this, however, was not significantly altered by any applied treatment (**Table 4-3**, page 148). As in wire-injured vessels, punctate Mac2 immunoreactivity was also present in all layers of the vessel wall (**Figure 4-8**) and similarly, was not modified by any combination of atrasentan and A192621 treatment (**Table 4-3**, page 148). Cell proliferation, as indicated by BrdU incorporation, was rarely detectable in either the media or the neointima of ligation-injured arteries (**Figure 4-8**) and again, levels were not different between experimental arms (**Table 4-3**, page 148).

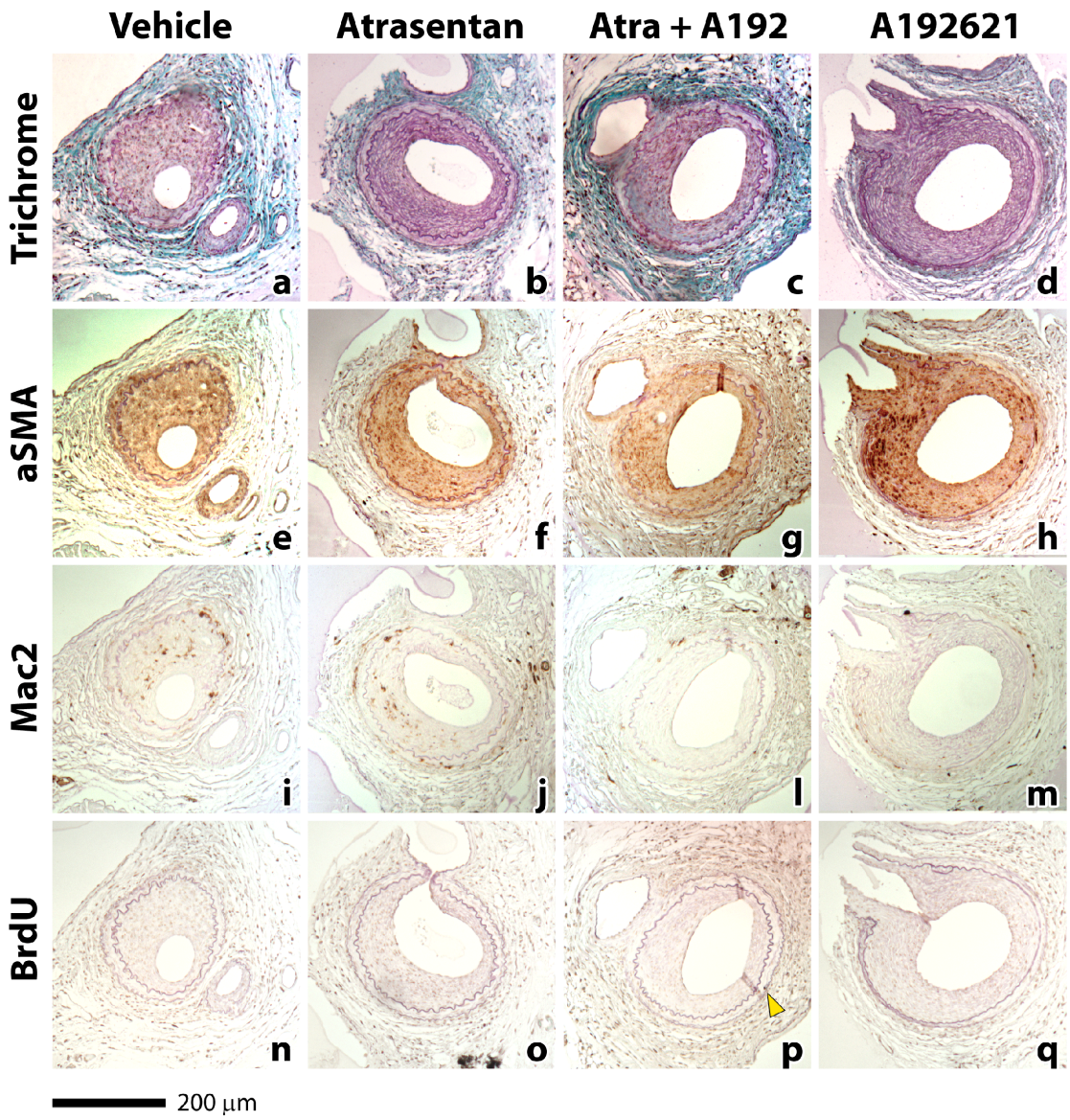


Figure 4-8 Impact of endothelin receptor blockade on complete femoral artery ligation injury-induced lesion size and composition.

Neointima formation was induced by complete femoral artery ligation-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before isolation and histological examination. Representative photomicrographs of lesions from all treatment groups stained with United States Trichrome (a-d), Picro-sirius red (n-q) and immunohistochemistries for αSMA (e-h) and Mac2 (i-m), are shown. Trichrome staining reveals formation of large, elastin (purple) and collagen (blue)-rich lesions which are similar in size in all treatment groups (a-d). All lesions are rich in immunoreactivity for αSMA which is also present in the media, but rarely in the adventitia (e-h). Mac2 immunoreactivity is also found throughout the vascular wall in injured vessels, although the extent of this is much less. BrdU immunoreactivity (yellow arrowhead) is rarely present in any layer of the vascular wall, and in lesions from any treatment group (n-q). αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine.

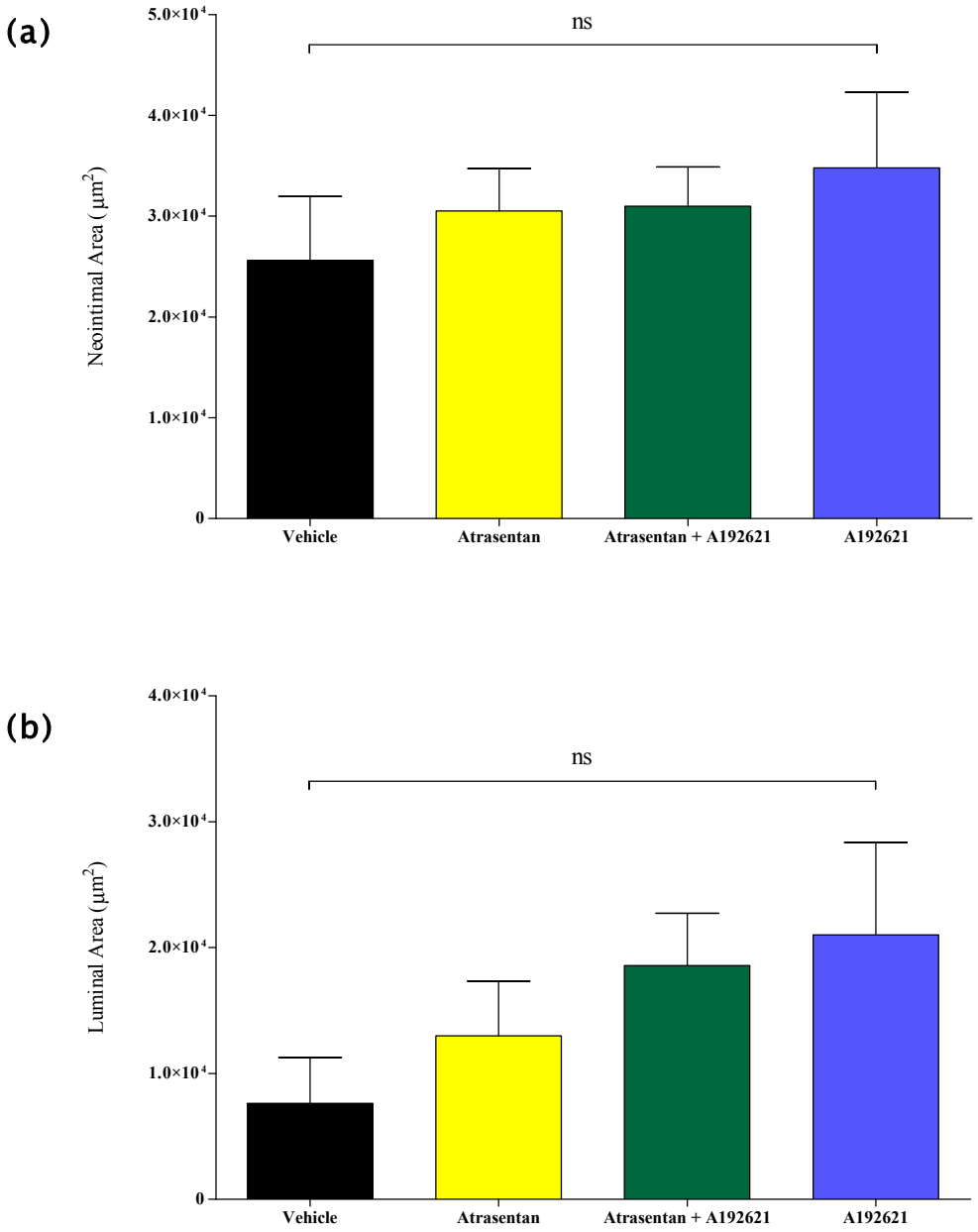


Figure 4-9 Impact of endothelin receptor blockade on neointimal and lumenal area in complete ligation-injured femoral arteries, as assessed by histology.

Neointima formation was induced by complete femoral artery ligation-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before histological examination. Neointimal (a) and lumenal cross-sectional areas (b) were measured at the point of largest lesion size by morphometry. Neointimal area was not significantly altered by any treatment (a). Similarly, lumenal cross-sectional area was not different between lesions from any treatment group. n=6-8. Data are mean ± SEM. ns, $p>0.05$ by two-way ANOVA with Dunnet's post-test.

Measurement	Vehicle	Atrasentan	Atrasentan +A192621	A192621
Body weight at study start (**g**)	24.5 ± 1.3	27.1 ± 0.8	27.3 ± 0.7	26.1 ± 0.7
Body weight at study end (**g**)	29.2 ± 1.3	31.6 ± 0.9	30.9 ± 0.6	29.7 ± 0.9
Change in body weight (**g**)	4.1 ± 0.4	4.5 ± 0.6	3.5 ± 0.4	3.9 ± 0.9
Food consumption (**g/day/mouse**)	10.3 ± 0.6	9.4 ± 0.5	10.3 ± 0.4	9.4 ± 0.5

Table 4-1 Impact of endothelin receptor blockade and femoral artery injury on body weight and food consumption.

Body weight and food consumption were measured in mice subject to femoral artery injury and treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621). Pre-study body weights were similar between all treatment groups. At the study end, body weights and change in body weight over the study duration did not differ between groups. Similarly, food consumption over the period of drug dosing was in-line with expected intake of 10g/day/mouse and not different between treatment groups. n=8-10. Data are mean ± SEM.

Measurement	Vehicle	Atrasentan	Atrasentan +A192621	A192621
(a) 3D Morphometry (OPT)				
n	6	7	8	6
Intimal volume (**x10^{7}µm^3**)	12.6 ± 0.9	3.6 ± 1.0	11.9 ± 3.4	23.5 ± 4.6
Lumenal volume (**x10^{7}µm^3**)	19.3 ± 2.2	25.3 ± 2.9	23.3 ± 2.5	11.5 ± 2.1
Volumetric stenotic ratio	0.40 ± 0.04	**0.12 ± 0.03****	0.30 ± 0.07	**0.65 ± 0.08***
(b) 2D Morphometry (Histology)				
n	9	7	8	7
Lumenal area (**x10^{3}µm^2**)	17.8 ± 5.5	**50.2 ± 8.9****	23.6 ± 5.8	12.4 ± 5.5
Intimal area (**x10^{3}µm^2**)	42.9 ± 3.6	27.0 ± 3.4	49.7 ± 6.9	**79.2 ± 11.5****
Medial area (**x10^{3}µm^2**)	14.4 ± 1.4	15.1 ± 1.1	12.3 ± 1.0	15.2± 2.1
Intima/Media ratio	3.1 ± 0.3	1.9 ± 0.5	4.3± 0.7	**5.4 ± 0.7***
Stenotic ratio	0.7 ± 0.1	**0.4± 0.1****	0.7± 0.1	0.9± 0.1
Area inside EEL (**x10^{3}µm^2**)	75.1 ± 6.9	93.1 ± 6.6	85.6 ± 4.7	**106.7 ± 12.6***
(c) Composition & Proliferation (Immunohistochemistry)				
n	9	7	8	7
Intimal αSMA IR(**%**)	46.5 ± 11.0	41.7 ± 9.6	30.4 ± 8.8	42.0 ± 8.3
Intimal Mac2 IR (**%**)	1.2 ± 0.3	1.4 ± 0.2	2.9 ± 1.2	2.9 ± 1.3
Intimal PSR staining (**%**)	54.9 ± 7.4	38.4 ± 5.0	**30.6 ± 8.7***	52.2 ± 5.7
Intimal BrdU IR (**count**)	1.3 ± 0.4	1.1 ± 0.4	1.1 ± 0.5	**5.1 ± 2.2 ***
Medial αSMA IR (**%**)	32.7 ± 7.5	32.9 ± 7.5	31.4 ± 8.1	32.6 ± 7.7
Medial Mac2 IR (**%**)	6.8 ± 2.0	6.4 ± 2.4	9.7 ± 3.2	14.3 ± 6.4
Medial BrdU IR (**count**)	0.5 ± 0.3	0.5 ± 0.3	0.8 ± 0.3	0.7 ± 0.4

Table 4-2 Impact of endothelin receptor blockade on the size and composition of femoral artery wire injury-induced neointimal lesions.

Neointima formation was induced by femoral artery wire-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before examination by optical projection tomography (a), histology (b) and immunohistochemistry (c). ETA blockade was associated with reduced lesion size and volume, and preserved lumenal cross-section without compositional changes. ETB blockade increased lesion size and volume, and the number of proliferating nuclei in lesions. The combination of ETA+ETB blockade did not alter lesion size or volume, but reduce lesion collagen content. Data are mean ± SEM. αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine; EC, endothelial cell. *, $p<0.05$; **, $p<0.01$ by two-way ANOVA with Dunnet's post-test vs. vehicle.

Measurement	Vehicle	Atrasentan	Atrasentan +A192621	A192621
(a) 3D Morphometry (OPT)				
n	5	5	7	6
Intimal volume (**x10^{7}µm^3**)	4.0 ± 0.9	1.7 ± 0.1	1.9 ± 0.3	3.9 ± 0.8
Luminal volume (**x10^{7}µm^3**)	4.9 ± 1.0	8.0 ± 1.5	8.0 ± 1.3	5.7 ± 0.9
Volumetric stenotic ratio	0.45 ± 0.09	**0.20 ± 0.04 ***	**0.20 ± 0.02 ***	0.41 ± 0.08
(b) 2D Morphometry (Histology)				
n	8	7	8	6
Luminal area (**x10^{3}µm^2**)	7.6 ± 3.6	12.9 ± 4.4	18.6 ± 4.2	21.0 ± 7.3
Intimal area (**x10^{3}µm^2**)	25.6 ± 6.3	30.5 ± 4.2	31.0 ± 3.9	34.8 ± 7.5
Medial area (**x10^{3}µm^2**)	10.8 ± 1.3	14.0 ± 1.1	13.3 ± 1.0	11.9 ±1.1
Intima/Media ratio	2.4 ± 0.4	2.5 ± 0.1	2.4 ± 0.3	2.9 ± 0.6
Stenotic ratio	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Area inside EEL (**x10^{3}µm^2**)	44.1 ± 10.7	62.3 ± 7.4	62.8 ± 8.1	67.7 ± 12.0
(c) Composition & Proliferation (Immunohistochemistry)				
n	8	7	8	6
Intimal SMA IR (**%**)	20.2 ± 7.0	16.4 ± 3.5	13.4 ± 2.3	26.2 ± 5.0
Intima Mac2 IR (**%**)	1.7 ± 0.3	2.0 ± 0.3	1.7 ± 0.5	1.4 ± 0.3
Intimal BrdU IR (**count**)	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.3 ± 0.2
MedialαSMA IR (**%**)	19.0 ± 3.7	13.2 ± 1.6	10.4 ± 1.1	21.2 ± 4.0
Medial Mac2 IR (**%**)	2.8 ± 0.5	3.6 ± 1.0	2.2 ± 0.2	2.9 ± 1.1
Medial BrdU IR (**count**)	0.3 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.2

Table 4-3 Impact of endothelin receptor blockade on the size and composition of complete femoral artery ligation-induced neointimal lesions.

Neointima formation was induced by femoral artery complete ligation-injury in mice treated with vehicle or antagonists to ETA (atrasentan; 10mg/kg/day), ETB (A192621; 30mg/kg/day) or ETA+ETB (atrasentan+A192621) and lesions allowed to develop for 28 days before examination by optical projection tomography (a), histology (b) and immunohistochemistry (c). Both ETA and ETA+ETB blockade reduced complete ligation-induced lesion volume fraction (a). This measure was not altered by ETB blockade. No treatment had any significant effect on histological measures of lesion size (b), or on lesion composition (c). Data are mean ± SEM. αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine; EC, endothelial cell. *, $p<0.05$ by two-way ANOVA with Dunnet's post-test vs. vehicle.

4.4 DISCUSSION

The experiments in this chapter were designed to determine the effects of systemic blockade of ET receptors on neointimal lesion formation following injury to the mouse femoral artery. Following intra-luminal, wire-induced injury, treatment with a selective ETB receptor antagonist was associated with an increase in lesion size, an observation which is in support of that of Murakoshi *et al* (2004) and suggests that the ETB acts primarily to limit neointimal growth. ETA blockade in contrast, reduced the size of lesions resulting from both femoral artery wire- and ligation-injury, further demonstrating the well-established role of ETA in exacerbating processes of neointima formation. Further, ETB blockade in ETA antagonist treated mice abolished the beneficial effects of the latter, clearly illustrating the ability of ETB activation to moderate the detrimental effects associated with stimulation of the ETA receptor by endogenous ETs. These studies also provide further validation to the use of the OPT imaging technique for measurement of vascular lesions.

4.4.1 *ETB blockade increases wire-injury induced lesion size*

Increases in several measures of wire injury-induced lesion size were apparent in mice treated with a selective ETB antagonist including maximal neointimal cross-sectional area as measured by histology and the fraction of the total vessel volume occupied by lesion, as determined by OPT. Importantly, these increases were present at 28 days post-injury, a time point known to reflect the maximum stable lesion size in this model (Sata *et al.*, 2000). As such, these data can be considered to represent an increase in stable lesion size rather than acceleration of lesion growth. Further, lesions from ETB antagonist treated mice exhibit a substantial increase in the number of actively proliferating cells (BrdU immuno-reactive nuclei following *in vivo* BrdU pulse). This increase in proliferation was of similar magnitude to that of lesion size (2.1-fold, 1.8-fold, respectively) and as such, may simply reflect the larger size of lesions in this group. Alternatively, an increase in cell proliferation may indicate that lesions continue to grow actively and therefore, it is the possible that in ETB antagonist treated animals, lesion size would continue to increase beyond 28 days. Whilst this proliferation is presumably of the smooth muscle-like cells that dominate

lesion content at this time point, without co-localisation studies, it cannot be ruled out that other cell types (such as macrophages) exhibit altered proliferation *in situ*.

Contrasting with the substantial increase in neointimal lesion dimensions observed in ETB antagonist treated animals, luminal dimensions remained similar to those in lesions from vehicle treated mice. This probably reflects the well-established phenomenon of compensatory outward 'Glagovian' remodelling of the vessel wall to accommodate increased lesion burden without restricting blood flow to distal sites, rather than specific modulation by ETB blockade (Glagov *et al.*, 1987). In agreement, the total area inside the external elastic lamina was increased, indicating that maintained luminal cross-section does not occur at the expense of atrophy of other layers of the arterial wall.

Whilst the mechanism by which ETB receptor blockade might increase lesion size in the femoral artery wire-injury model was not explicitly investigated, (immuno)-histochemical studies of lesion composition may offer some indications. The fraction of lesion occupied by smooth muscle-like cells and collagen, two major lesion constituents at this time point (Sata *et al.*, 2000), was not altered by ETB blockade. This suggests that ETB blockade does not alter lesion size by specifically regulating collagen synthesis or metabolism and may also indicate that an action on smooth muscle cells can be discounted. The latter, however, is less certain because any increase in smooth muscle cell content is likely to be associated with increased extracellular matrix deposition thus blunting any effect on *fractional* composition. ETB blockade was also associated with a non-significant trend in the fraction of neointima (but not media) that exhibited Mac2 immunoreactivity. This may indicate that an enhanced inflammatory response, or defects to its resolution, underlies the increase in lesion size. This is certainly plausible given the central role of the macrophage in lesion formation, but further studies are required to confirm this observation. Indeed, the studies reported here investigate the 28 day time point so as to best address the primary end point, lesion size. However, by definition, this time of stable lesion size poorly represents the processes of lesion development including

cell proliferation and macrophage content. As such, detailed studies of these processes would require analysis of lesions at earlier time points of lesion development (7 and 14 days).

Proposing the mechanism by which ETB can moderate neointima formation is difficult because numerous cell types from which it is composed, including smooth muscle cells, macrophages and endothelial cells, have been shown to express ETB, both *in vitro* and, in neointimal lesions, *in vivo* (see section 1.3.2). A primary hypothesis of this thesis is that such an effect is a product of stimulation of the EC ETB receptor by endogenous ET-1. Certainly, the vasodilator response to EC ETB activation is well known, and cultured endothelial cells from a variety of species (Hirata *et al.*, 1993; Tsukahara *et al.*, 1994), including mouse (Azfer *et al.,* Unpublished observations), release nitric oxide and up-regulate expression of eNOS in response to ETB activation. The potent inhibitory effect of endogenous NO on smooth muscle proliferation and macrophage activation is also clear (Moroi *et al.*, 1998; Le Tourneau *et al.*, 1999; Zhang *et al.*, 2006). ET-1, abundantly produced within vascular lesions, may, therefore, act upon the EC ETB pathway to stimulate NO release and moderate development of the underlying lesion. Thus, blockade of this protective system might mediate the increase in lesion size observed in A192621-treated animals following femoral artery wire-injury. This explanation is certainly consistent with the conclusions of Murakoshi *et al* (2004) who reported decreased levels of NO oxidation products in the carotid artery ligation-induced lesions of ETB-deficient mice. Further, reduced NO production might explain both the trend towards increased intimal macrophage content and the increase in cell proliferation observed in wire-injured femoral arteries from ETB antagonist-treated mice.

Any explanation of the effect of ETB blockade on lesion formation in the femoral artery wire-injury model depending on endothelial cells might be considered implausible because wire-insertion effectively removes the endothelium (Sata *et al.*, 2000). As such, there may be too few ECs remaining on which ET-1 can act to alter

lesion development. Some effect on those EC not directly overlying the site of lesion formation may still be plausible such as those at the fringes of the denuded length or those lining the adventitial *vaso vasorum*, which is abundant in injured vessels. A more compelling argument, however, is that the luminal endothelium can recover sufficiently quickly that modulation of its function can impact on underlying lesion formation. Previous work with this model in this laboratory indicates that complete luminal coverage, by a monolayer of cells with endothelial-like morphology and immuno-reactivity for von Willebrand factor, returns between 7 and 14 days following injury (MacDonald *et al*., Unpublished data). As discussed (chapter 3), assessment of the function of this neo-endothelium has been prevented by the severe medial damage caused by wire insertion, although data from similar models suggests that substantial endothelium-dependent responses may recover with a similar time course (Miller *et al.*, 2003; Liao *et al.*, 2007). Importantly, this time point precedes the majority of neointimal smooth muscle accumulation and lesion growth (Sata *et al.*, 2000). Therefore, in murine models similar to femoral artery wire-injury, recovery of the function of the luminal endothelium may be rapid enough to allow for endothelium-mediated modulation of lesion development.

The ability of ETB to regulate lesion size in the femoral artery wire-injury model, despite endothelial denudation, still raises the intriguing possibility of an endothelium-independent mechanism of action. The literature would suggest that the primary actions of ETB activation on non-endothelial cell types (vascular smooth muscle cells, fibroblasts, macrophages) are those that might enhance lesion formation. For example, in culture, ETB stimulates proliferation of various smooth muscle and fibroblast cell lines, chemotaxis of macrophages and their release of pro-inflammatory and mitogenic mediators (see section 1.3.1). One possibility is that ETB blockade can increase lesion size by reducing smooth muscle cell apoptosis. Such a pro-apoptotic effect of ETB has been observed in cultured VSMCs exposed to cyclic stretch (Cattaruzza *et al.*, 2000), and arterial rings subject to brief over-pressurisation (Lauth *et al.*, 2000). It might also be considered that the phenotype of cells participating in neointima formation does not reflect that seen in cell culture or other *in vivo* models. Intimal smooth muscle cells in femoral artery wire-induced

lesions, for example, have been shown to express eNOS and iNOS (Wang *et al.*, 2005a). It is plausible, therefore, that, in VSMC as in EC, ETB could stimulate release of NO, a powerful inhibitor of neointimal proliferation (Moroi *et al.*, 1998; Le Tourneau *et al.*, 1999).

4.4.2 *ETB blockade does not increase lesion size by a systemic action?*

An indirect mechanism of action, in which ETB receptors reduce lesion size by regulation of systemic cardiovascular parameters, is also plausible. A modest elevation in blood pressure was observed in ETB antagonist treated mice and this is consistent with previous studies showing chronic ETB blockade (Fryer *et al.*, 2006) or ETB knockout is associated with hypertension (Gariepy *et al.*, 2000). Whilst this provides useful confirmation of the effective administration of A192621, hypertension is associated with worsening of many vascular remodelling processes including medial hypertrophy, atherosclerosis and possibly restenosis following percutaneous coronary interventions (Gibbons & Dzau, 1994; Klugherz *et al.*, 2000; Singh *et al.*, 2005; Feihl *et al.*, 2008). The increase in wire injury-induced lesion size observed in ETB antagonist treated mice might, therefore, simply be a response to the hypertensive action of this treatment. The strongest evidence against this supposition is that even in the presence of concurrent ETA blockade, ETB antagonist treatment was still associated with a relative increase in lesion size (as compared to ETA blockade alone) despite an absence of further blood pressure alteration. Moreover, data from α_{1D}-adrenoceptor knockout mice also indicate that, in the femoral artery wire-injury model, alterations in blood pressure of the order seen here (~10mmHg) do not inevitably result in reciprocally-altered lesion development (Hosoda *et al.*, 2007).

A second systemic role for ETB, blockade of which might explain an increase in lesion size in mice treated with A192621, is the clearance of circulating ETs. A wealth of studies indicate that blockade or deletion of ETB increases plasma ET-1 concentration due to loss of this important receptor-mediated clearance pathway (Fukuroda *et al.*, 1994a; Giller *et al.*, 1997). Given the obviously detrimental effect

of ET-1 on lesion formation, it may be that ETB blockade enhances lesion formation by increasing the activation of the ETA receptor by endogenous ET-1, as a direct consequence of this impaired clearance. The ability of ETB blockade to reduce lesion size even in the presence of concurrent ETA blockade, again suggests that this systemic action of ETB blockade does not underlie the observation. Indeed, the dose of atrasentan administered is reported sufficient to abolish the pressor response to pharmacological amounts of ET agonists: doses of far greater magnitude than those that might be predicted to result from impaired clearance of endogenous ETs (Opgenorth *et al.*, 1996).

4.4.3 *ETB does not regulate femoral artery ligation-induced neointima formation*

The striking effects of ETB blockade on neointima formation in the femoral artery wire-injury model must be tempered by the observations that, following femoral artery ligation-injury, selective ETB blockade has little effect on any parameter of the neointima. Such a finding was surprising given that A192621-treatment and ETB-deletion substantially increase lesion size following ligation of the carotid artery (Murakoshi *et al.*, 2002). This may reflect inherent differences between carotid and femoral arteries in either the ET system or the mechanism of neointima formation. ET-1-mediated vasoconstriction in the carotid artery is certainly less dramatic than that observed in the femoral artery (see chapters 3 and 5; Widmer *et al.*, 2006; Bhattacharya *et al.*, 2008). This discrepancy may also reflect the stage of lesion development analysed. Murakoshi *et al* (2002) examined ligated carotid arteries for lesions 14 days after surgery, a time of active growth and intermediate lesion size (Godin *et al.*, 2000). In contrast, here arteries were examined 28 days post-ligation, a time at which lesion size appears to have peaked (see chapter 5). Thus, following both femoral and carotid arterial ligation, lesion growth may be accelerated - the endpoint investigated by Murakoshi *et al* (2002) rather than maximally increased - the endpoint investigated here.

The divergent effects of ETB antagonism on wire- and ligation-induced injuries are also difficult to explain. As both injuries were performed in contra-lateral arteries of

the same animals, any doubts regarding effective drug administration (or ETA/ETB selectivity) can be dismissed. The numerous differences between the two models complicate interpolation of a differential mechanism of action, although several possibilities exist, including the absence of physical damage to the endothelium. It is plausible that, following vascular injury, ET stimulates EC to produce oxidative species (Duerrschmidt *et al.*, 2000; Dong *et al.*, 2005) or exert other potentially lesion enhancing actions. Removal of these influences by wire-injury might, therefore, reveal a beneficial effect of ETB on other cell types. Moreover, although the endothelium is intact throughout the period of lesion development in the ligation model, prior studies (Chapter 3) indicate it to be moderately dysfunctional at 28 days. Further, more severe defects may be present at earlier time points at which lesions are actively developing. As such, despite physical integrity, it may be that the endothelial cell ETB/ NO production pathway is functionally impaired in this model. It is also pertinent that in conditions of endothelial dysfunction, as induced by tetrahydrobiopterin deficiency, stimulation of NOS activity by ETB results in generation of superoxide (Loomis *et al.*, 2005), a smooth muscle mitogen and scavenger of NO that may contribute to lesion growth.

The origin of the smooth muscle cells in wire- and ligation-induced injuries may also warrant consideration as a mechanism of differential sensitivity to ETB blockade. Studies of femoral artery wire-injury following bone-marrow transplant indicate that between 20% and 80% of αSMA immunoreactive cells in the neointima are derived from circulating progenitors. In contrast, in both carotid artery ligation and femoral artery cuff placement models circulation-derived smooth muscle-like cells do not contribute to lesion formation (Tanaka *et al.*, 2003). In principle, at least, ETB blockade may either alter recruitment of these cells, or elicit unpredictable responses in them, explaining the divergent responses to this treatment in these two models. Whilst this is purely speculative, reductions in vascular NO, perhaps as induced by ETB blockade, are associated with increased circulating and adventitial levels of putative smooth muscle cell progenitors in $eNOS^{-/-}$ mice (Zhang *et al.*, 2006). A final possibility for discussion is that ETB attenuates the response to wire-induced lesion formation purely by inhibition of stretch-induced VMSC apoptosis. If true, it follows

that the ligation model would be insensitive to ETB blockade because no comparable apoptosis-inducing stimulus is present here.

4.4.4 *ETA blockade inhibits wire- and ligation-induced lesion formation*

In both femoral artery wire-injury and ligation models, selective ETA blockade was seen to reduce lesion formation suggesting the role of ETA in the response to vascular injury is less dependent on the etiology of the initiating insult. This finding is supported by much literature and the principle that ETA activation enhances the processes of neointimal hyperplasia (see **Table 1-3**, page 48). Given the muscular nature of the mature neointimal lesion, a compelling explanation for this effect is the well described (co)-mitogenic action of ETA receptor activation on vascular smooth muscle cells. Immunohistochemical analyses of lesion composition do not directly support this as neither αSMA immunoreactivity nor proliferating (BrdU incorporating) cell count was altered by atrasentan treatment in either model. As described above for ETB blockade, however, because VSMCs may drive deposition of other major lesion constituents (particularly extracellular matrix components), an inhibitory action on smooth muscle cells may not necessarily translate into a reduction into *fractional* VSMC content. In wire-injured vessels from ETA antagonist treated mice, a trend towards reduced fractional collagen content was seen. This may reflect reduced collagen deposition or increased activity of collagenolytic MMPs - both processes that have been observed to result from ETA activation *in vitro* (Shi-Wen *et al.*, 2004; Gallelli *et al.*, 2005). Although ETA is also implicated in fibroblast proliferation any inhibition of this process might be expected to alter proliferating cell count and immunoreactivity for αSMA, an antigen also expressed by myofibroblasts.

4.4.5 *ETA and ETB blockade have additive effects on lesion size*

The combination of ETA and ETB antagonist treatment did not alter any parameter of wire injury-induced lesion size at the time point investigated. This is in contrast to the effect of either treatment when administered alone. Differing levels of ETA blockade between ETA and ETA+ETB treatment arms cannot explain this

observation, as identical doses of atrasentan were administered in both groups. Given the magnitude of individual responses to ETA and ETB blockade, it is likely that this absence of effect of their combination reflects a simple composite of two functionally antagonistic responses i.e. ETA blockade-mediated inhibition and ETB blockade-mediated enhancement of lesion size. In support of this notion, in the ligation-injury model, combined ETA+ETB blockade was seen to reduce lesion volume to a similar extent as ETA blockade alone. This, therefore, also describes a composite of the response to selective ETA and ETB blockade as ETB blockade is without effect on lesion size in this model. As such, the alternative explanation of a direct interaction whereby ETB receptor activation is required for ETA blockade to reduce wire-injury (but not ligation injury-induced) lesions is less likely. Such an interaction is plausible, however, as complex interactions between ET receptor subtypes have been proposed such as formation of ETA:ETB heterodimers which may be important in determining receptor pharmacology (Gregan *et al.*, 2004). Regardless, these conflicting responses between ligation- and wire-induced injuries also mirror the inconsistent responses to ETA/B blockade on neointimal lesion development reported in the literature (McKenna *et al.*, 1998; Huckle *et al.*, 2001). As both injury models have been performed in the same animals here, this suggests that such variability reflects a genuine dependence of the response to this treatment on the type of injury inflicted, rather than differences in experimental execution or design, such as the particular ET antagonist(s) used. Moreover, this comparison of ETA and ETA+ETB blockade is of relevance to man as both treatment modalities are in clinical use and being investigated for a variety of other cardiovascular indications (see chapter 6 for discussion). These data may, therefore, indicate that selective ETA antagonists have additional benefits to vascular health, which are lost in the presence of concurrent ETB blockade.

Analysis of the composition of lesions from mice treated with both ETA and ETB antagonist are also broadly in line with the suggestion that lesion phenotype in this treatment group is a composite of that seen when either antagonist is administered alone. In ligation injury induced lesions, no compositional abnormalities were observed in any treatment group. In wire injury-induced lesions, as for selective ETA

and ETB blockade, lesion αSMA immunoreactivity was unaltered by ETA+ETB blockade, and as for selective ETB blockade, a trend towards increased Mac2 immunoreactivity was apparent. Lesion collagen content, however, was significantly reduced by combined ETA+ETB blockade but neither treatment alone (a trend towards reduction was present with selective ETA blockade). This may be explained by the published literature that consistently indicates that whilst ET-1-stimulated fibroblast activation can be inhibited by the combination of ETA and ETB blockade, it is not sensitive to either treatment alone (Guarda *et al.*, 1993; Cambrey *et al.*, 1994; Xu *et al.*, 1998; Shi-Wen *et al.*, 2001; Clozel & Salloukh, 2005).

4.4.6 *OPT for vascular imaging*

In addition to addressing hypotheses regarding the role of ET receptor subtypes in the response to acute vascular injury, these experiments further highlight the potential of the OPT imaging technique for quantification of vascular lesions. In wire-injured femoral arteries, measures of neointima size produced from OPT scans faithfully represented the effect of treatment indicated by traditional histological means i.e. both histological and OPT-derived measures of the neointima were increased by ETB blockade and reduced by ETA blockade. Treatment-induced changes in luminal volumes appeared to be less representative of those to luminal cross-sectional area as determined by histology, however. This probably reflects an unavoidable consequence of the methodology of quantification: a constant length of vessel must be considered across all arteries studied, thus, in most cases, measures of luminal volume describe large regions of vessel which are not afflicted by neointima formation. As such, any impact of the response to vascular injury on luminal volume is likely to be strongly diluted, whereas, by contrast, the finite nature of the neointimal layer makes it relatively insensitive to such concerns.

OPT measurements of neointimal volume in ligation-injured vessel highlight the potential importance of considering changes to vessel morphology in three dimensions. In this model, treatment regimens that included ETA blockade were seen to reduce neointimal volume without any apparent effect on the cross-sectional

area of neointima at its greatest point, the quantity conventionally recorded during histological examination. Analysis of the cross-sectional profile of lesion formation provided by OPT revealed that rather than a genuine discrepancy between measurement techniques, the effect of treatment was to reduce the length of vessel afflicted by neointima. This effect would not have been observed had vessels been analysed only by traditional means. Thus, the application of the OPT technique to the studies of vascular injury presented in this chapter has further highlighted its value for rapid, non-destructive imaging of neointimal lesion formation, and has provided additional insight into the effects of ET antagonists on lesion formation which might otherwise have been missed.

4.4.7 *Conclusions*

The data presented in this chapter provide critical proof of concept for the role of the ETB receptor in the response to vascular injury, strongly supporting the hypothesis that the ETB receptor represents an endogenous mechanism for limiting the otherwise exuberant proliferative response to acute vascular injury induced by ETs. The detrimental response to ETB blockade following wire-injury is unlikely to be a result of systemic actions such as elevated blood pressure and circulating ET-1 concentration, but the mechanism is otherwise not yet clear. These straightforward conclusions are also complicated by the inability of ETB blockade to alter ligation-induced lesion development in the femoral artery. Whether this indicates the unimportance of ETB in this model, or a dichotomous role for this receptor, mediating both lesion promoting and diminishing effects in competition, also remains to be addressed. Data from both models support the well-established view that blockade of ETA might be useful in the clinical prevention of vascular remodelling processes. Further, given the possibility that concurrent ETB blockade might abrogate this effect, this work supports the argument in favor of selective ETA rather than non-selective ETA/B antagonists in this setting.

Chapter 5

Effects of endothelial cell-specific endothelin-B receptor deficiency on neointimal lesion formation

5.1 INTRODUCTION

The previous chapter described the ability of pharmacological blockade of the endothelin-B receptor to exacerbate neointimal hyperplasia following acute vascular injury. This effect serves to confirm the observations of Murakoshi *et al* (2002) and highlights a role of the ETB receptor as a mechanism to moderate the detrimental actions of endogenous ET-1 on the ETA receptor following vascular injury. Any understanding of the mechanism of this action, however, is complicated by the expression of ETB by many of the cell types important to neointimal lesion development, including vascular smooth muscle cells (VSMC), leucocytes, fibroblasts and endothelial cells. Neither pharmacological nor classical gene knockout approaches are able to distinguish between the diverse actions mediated by ETB on these different cell types. To address this issue, a cell-specific ETB knockout system was previously developed in our laboratory using Cre-lox technology (Bagnall *et al.*, 2006).

ETB receptors expressed in the endothelium may be important regulators of vascular function and the response to vascular injury. Certainly, in most species, bolus administration of ET agonists stimulates transient hypotension before prolonged vasoconstriction (de Nucci *et al.*, 1988; Yanagisawa *et al.*, 1988; Whittle *et al.*, 1989; Gardiner *et al.*, 1990; Clozel *et al.*, 1992; Rogerson *et al.*, 1993; Haynes *et al.*, 1995). Similarly, ETB activation stimulates endothelium-dependent vasodilatation in isolated arteries (Takayanagi *et al.*, 1991; Clozel *et al.*, 1993), and nitric oxide (NO) production in cultured endothelial cells (Hirata *et al.*, 1993; Tsukahara *et al.*, 1994). The mouse is unusual in that the response to an intravenous bolus of ET-1 comprises only vasoconstriction without prior vasodilatation (Giller *et al.*, 1997). In isolated mouse aortas, however, ETB-stimulated vasodilatation has been reported in several (Mizuguchi *et al.*, 1997; Bagnall *et al.*, 2006) but not all studies (Zhou *et al.*, 2004). Further, aortas from both transgenically-rescued, non-cell-specific ETB knockout mice (Quaschning *et al.*, 2005) and EC-specific ETB-deficient mice, exhibit modestly impaired endothelium-dependent vasodilatation, even to non-ET agonists (Bagnall *et al.*, 2006). These models also feature elevated plasma ET-1 concentrations, highlighting the importance of ETB in endothelial cells in the

clearance of circulating ET-1 (Fukuroda *et al.*, 1994a; Bagnall *et al.*, 2006). As NO release is known to suppress (Moroi *et al.*, 1998), and ET-1 release to enhance, neointima formation (Douglas *et al.*, 1994), ETB expressed by endothelial cells may be important in limiting lesion growth following vascular injury. Specifically, reduced NO production and increased ET-1 levels may explain why in the previous chapter, pharmacological ETB blockade increased the size of femoral artery wire injury-induced neointimal lesions. Indeed, Murakoshi *et al* (2002) report that the increase in carotid artery ligation-induced neointima formation observed in ETB-deficient mice is associated with reduced levels of NO oxidation products.

This chapter addresses the hypothesis that disruption of the ETB receptor gene selectively in endothelial cells is sufficient to increase neointimal lesion size following acute vascular injury as a consequence of impaired endothelium-dependent vasomotor function. Therefore, the specific aims of this work were to determine:

a) whether EC ETB deficiency increases neointimal lesion size following femoral artery wire- and ligation-injury
b) the effects of EC ETB deficiency on femoral artery vasomotor function in health and in situations of endothelial dysfunction (induced by ageing).

5.2 METHODS

5.2.1 *Generation of EC-specific ETB knockout mice*

The studies presented in this chapter were performed using EC-specific ETB receptor knockout mice, generated by the cross of homozygous floxed ETB mice (FF/--) with transgenic Tie2-Cre mice (Kisanuki *et al.*, 2001). The vast majority of the resulting progeny were of the genotypes FF/tie2 (EC-specific ETB knockout) and FF/-- (floxed, Cre-negative). In line with previous studies with these animals and other work utilising the Cre-loxP recombination system (Liao *et al.*, 2001; Ge *et al.*, 2006), floxed Cre-negative littermates (FF/--) were used as experimental controls (Bagnall *et al.*, 2006; Kelland et al., Unpublished data).

5.2.2 *Genotyping*

PCR of genomic DNA isolated from ear clip tissue samples and gel electrophoresis of the resulting products was used to identify the genotype of experimental animals. The presence of the Tie2-Cre transgene was indicated by a single ~550bp amplicon when PCR was performed using primer oligonucleotides 5'-CGC ATA ACC AGT GAA ACA GCA TTG C-3' and 5'-CCC TGT GCT CAG ACA GAA ATG AGA-3' (Kisanuki *et al.*, 2001). The genotype of the ETB allele was determined to confirm the presence (FF/tie2) or absence (FF/--) of recombination of the floxed loci. Products of PCR using the primers 5'-TCA GTT GTA ATG AGA CAC AGA C-3' and 5'-AGC CAT AAA GTC ACA GCC ATT C-3' included a ~1kb amplicon 'F' representing the floxed, non-recombined ETB allele, and a ~200bp amplicon '0' representing the recombined, non-functional allele (Bagnall *et al.*, 2006). As DNA extracted from ear clips contained both endothelial and non-endothelial cell types, in FF/tie2 animals, both F and 0 products were expected.

5.2.3 *Vascular injury studies*

5.2.3.1 *Femoral artery injury*

Femoral artery injury was performed as described (section 2.3.1) in 2-4 month old male FF/tie2 mice and FF/-- littermates. For study of lesions at 28 day after injury,

the time point investigated with ET antagonists (chapter 4), wire-injury was performed on the left femoral artery. To the right femoral artery of the same mice, partial ligation (ligation of the femoral artery immediately distal to the femoropopliteal bifurcation) was conducted. As discussed (chapter 3) neointimal lesions resulting from this treatment proved to be extremely variable and focal. Thus, data from this study arm were not analysed. For investigation into the effects of complete femoral artery ligation (ligation of the femoral and popliteal arteries across their junction) in EC ETB knockout mice at 28 days, ligation was performed on the left femoral artery only of a further group of mice, in which the right femoral artery remained uninjured to allow the study of baseline morphology and composition.

To address the possibility that EC ETB knockout might alter the rate of lesion development, and to better analyse macrophage content and cell proliferation, additional studies of wire/ligation-injury at 14 days after surgery, a time-point of active lesion growth, were carried out. Here, wire-injury was inflicted to the left femoral artery and complete ligation to the right femoral artery of the same mice.

Following the appropriate surgical recovery time (14 or 28 days), mice were killed by transcardiac perfusion fixation under terminal anesthesia. In 14 day wire/ligation injury mice and 28 day complete ligation injury mice, 100mg.kg^{-1} 5-bromo-2-deoxyuridine was administered by the intra-peritoneal route 2 hours prior to the induction of terminal anesthesia. Vessels were isolated, formalin fixed and their identity blinded for further analysis.

5.2.3.2 *Optical projection tomography (OPT)*

OPT scans and reconstructions of injured femoral arteries were produced according to the methods set out in section 2.5. Briefly, optically cleared tissues were scanned in a Bioptonics 3001 tomograph under metal-halide lamp illumination (excitation filter: 425/40nM; emission filter: LP475nM). 1.048Mpixel resolution projection images were captured at 0.9° rotation increments and reconstructed using NRecon software. From these, intima/media borders were manually traced and the area within

grey-level thresholded to delineate neointimal from luminal volume. OPT scans were not acquired from either uninjured or 28 day wire-injured femoral arteries.

5.2.3.3 *Histological evaluation*

Femoral arteries from all studies were subject to morphometric assessment. Following infiltration and embedding in paraffin wax, 3μm thick sections were cut perpendicular to the axial plane of each vessel. Sets of 16 (wire-injury) or 10 (ligation-injury) serial sections were retained at intervals of 100μm (wire-injury) or 50μm (ligation-injury) along the vessel until the lesion-afflicted portion had been traversed. From each set, 1 slide was stained according to the United States trichrome method (see section 2.4.2) and the largest lesions from each vessel identified. Photomicrographs were recorded under 10X objective magnification, and from these, measurements of neointimal area, luminal area, medial area, intima/media ratio and stenotic ratio were made. In uninjured vessels, media/lumen ratio was also calculated to assess the presence of medial hypertrophy.

5.2.3.4 *Immunohistochemistry*

Lesion composition and cell proliferation in neointimal lesions were assessed by immunohistochemistry for cell-specific antigens (α-smooth muscle action; αSMA and Mac2) and incorporated BrdU. Staining was performed on sections adjacent to those from which measurement of morphometric parameters was made and carried out according to the labelled streptavidin-biotin method. Antibody complex binding was visualised by the peroxidase-catalysed development of 3,3-diaminobenzidine. For αSMA and Mac2, immunoreactivity was quantified by colour segmentation of photomicrographs according to definitions defined from positive control slides. Immunoreactive area was expressed as a percentage of total area of the neointima or media. BrdU staining was quantified by manual count of immunoreactive nuclei.

5.2.4 *Vascular function studies*

5.2.4.1 *Tissue and myography preparation*

Experiments to determine vascular function were undertaken in vessels taken from FF/tie2 and FF/-- mice (young: 2-4 month and aged: 7-10 month) killed by CO_2 asphyxiation. Left and right femoral arteries were excised, cleaned of peri-adventitial tissue and divided into ~2mm rings. Aortic rings were taken from the first ~2mm immediately distal to the curvature of the aortic arch and similarly cleaned of fat and connective tissue. Vessels were mounted on parallel intra-luminal wires in a small-vessel myograph (Danish Myo Technology 610M) and bathed in physiological salt solution (PSS). Resting tension of 8mN was gradually applied to both vessel types, after which isometric tension developed by each vascular ring was recorded.

5.2.4.2 *Common myography protocol elements*

In all vessels, contractility was initially assessed by repeated (3) exposure to iso-osmolar, 125mM KCl PSS (KPSS) before recording cumulative concentration response curves (CRCs) to phenylephrine (PE, 10^{-9}–10^{-4}M, 2 min addition cycle) and acetylcholine (10^{-9}–$3x10^{-5}$M, 1 min addition cycle). Responses to acetylcholine (ACh) and all other dilator agents were measured in vessels pre-contracted with a concentration of phenylephrine producing 80% of maximal constriction. Following these common elements, two main protocols were used to investigate vascular function, each culminating in the measurement of a response to an ET agonist. The prolonged nature of ET-stimulated vasoconstriction (Yanagisawa *et al.*, 1988; Widmer *et al.*, 2006) and the resistance of S6c-treated vessels to further ETB agonist stimulation (Lodge *et al.*, 1995) necessitated the use of a separate femoral artery ring for every combination of ET agonist and antagonist. In aortic rings, experiments were carried out according to protocol 2, except that responses to S6c were not assessed.

5.2.4.3 *Myography protocol 1 – ET-1/S6c studies*

After washing ACh from the tissue, a CRC to KPSS (containing 5-125mM KCl with reciprocal molar reduction of [NaCl]; 2 min addition cycle) was recorded. Each

concentration was achieved by replacement of the bathing solution with of an appropriate mixture of PSS and KPSS. After further washing, a CRC to sodium nitroprusside (SNP; 10^{-9}–$3x10^{-5}$M; 2 min addition cycle) was measured in PE pre-contracted rings and thoroughly washed out. In all vessels this protocol was completed by recording either (i) responses to ET-1 (10^{-11}-$3x10^{-7}$M, 3 min addition cycle) after a 30 minute incubation with, where appropriate, BQ123 (10^{-6}M), A192621 (10^{-7}M) or vehicle or (ii) by assessing the effect of a single dose of sarafotoxin S6c (10^{-7}M) in PE pre-contracted rings.

5.2.4.4 *Myography protocol 2 – L-NAME/S6c studies*

After washing ACh from the tissue, vessels were incubated with the NOS inhibitor L-NAME ($2x10^{-4}$M) for 30 mins before washing, re-addition of L-NAME and measurement of an additional CRC to ACh. After further washing, L-NAME was re-added to the tissue bath, and the response to a single dose of S6c (10^{-7}M) recorded.

5.2.5 *Statistics and data analysis*

All data describing vascular morphology and composition in mouse femoral arteries, and the response to S6c were statistically-analysed by Student's un-paired t-test between FF/tie2 and FF/-- group. Where multiple time points were investigated, only the effect of genotype was the basis of statistical testing. Because absolute contractility to KPSS did not differ between genotypes (FF/--, 5.94±0.35 $mN.mm^{-1}$; FF/tie2, 5.60±0.54 $mN.mm^{-1}$; p=0.59), all other vasoconstrictor responses were expressed and analysed as percentage of the maximum response to KPSS for that vessel, to reduce variability associated with trauma to the vessel during preparation. Myography CRCs were analysed by two-way ANOVA between FF/tie2 and FF/-- groups within each treatment. These data were additionally fitted to a logistic equation to derive summary data of E_{max} (maximum response) and $logEC_{50}$ (concentration resulting in 50% maximal response). These too were subsequently analysed by Student's unpaired t-test between genotypes. Where the same response was measured in multiple rings from one animal, the mean value was treated as n=1. All analyses were performed using Prism 4.0 software (Graphpad software, USA).

5.3 RESULTS

5.3.1 *Genotype*

Deletion of the endothelial cell ETB receptor was confirmed by genomic PCR of ear clip DNA (**Figure 5-1**). FF/tie2 animals tested positively for Tie2-Cre and exhibited floxed and recombined ETB alleles, reflecting genotype of endothelial and non-endothelial cells in the test tissue, respectively. In FF/-- animals, neither Tie2-Cre, nor recombination of the ETB allele was observed.

5.3.2 *Physiological parameters*

No abnormalities of litter size, development or body weight (either pre- or post-operative) were noted in endothelial cell specific ETB knockout mice (FF/tie2), as compared to floxed, Cre-negative littermates (FF/--). Wet weight of isolated organs (heart, liver, kidneys) was also not altered by EC-ETB deficiency in either age group (**Table 5-1**, page 186).

5.3.3 *Un-injured femoral artery morphometry and composition*

Un-injured femoral arteries from FF/tie2 and FF/-- animals were of similar appearance when examined by routine histology (**Figure 5-2**). Measurements of medial area ($p=0.156$), luminal area ($p=0.948$) and media/lumen ratio ($p=0.507$) were not different between these genotypes (**Table 5-2**, page 187). Immunoreactivity for α-smooth muscle actin in the medial layer was also similar between uninjured femoral arteries from FF/-- and FF/tie2 mice (**Figure 5-2**; $p=0.121$).

5.3.4 *Wire injury*

5.3.4.1 *3D Morphometry*

OPT imaging of wire-injured femoral arteries, as previously described (chapters 3 and 4), clearly indicated the presence of neointima formation at 14 days. Volumetric analysis, however, indicated no effect of genotype on either neointimal volume ($p=0.557$) or volumetric stenotic ratio ($p=0.281$) at 14 days (**Table 5-3**, page 188). A

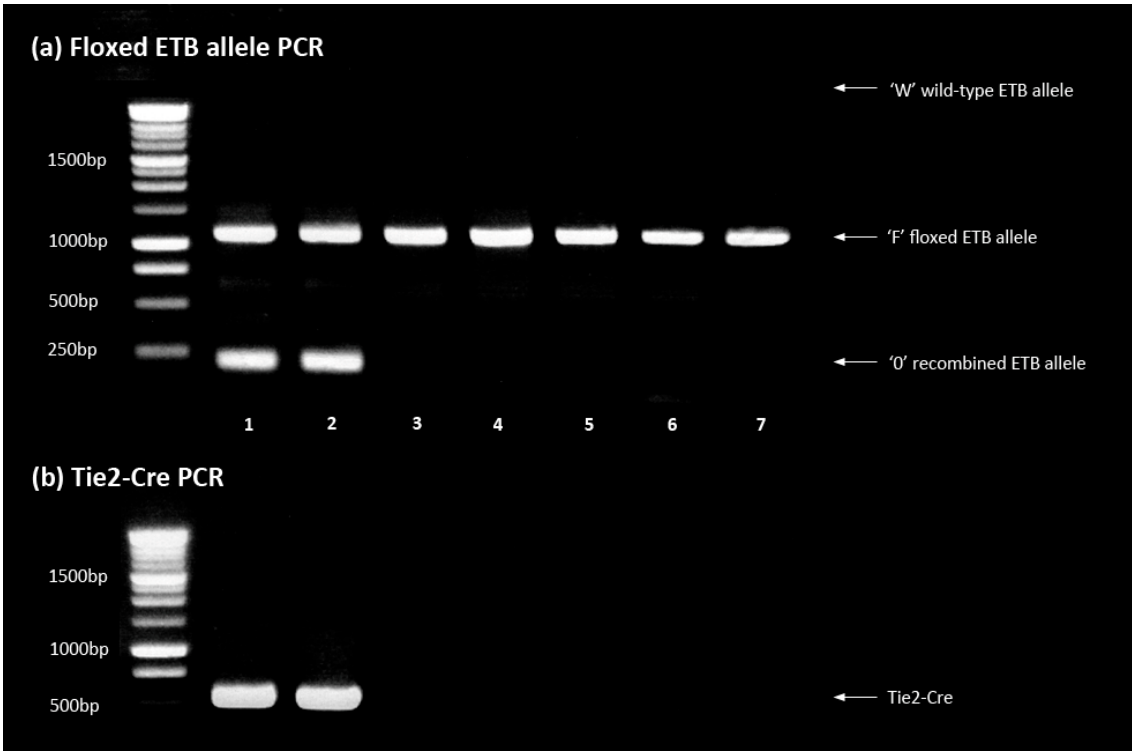


Figure 5-1 Electrophoresis-separated products of EC ETB-deficient mouse genotyping PCRs.

Genotyping of EC ETB-deficient mice was routinely performed using PCR primers spanning exons 2 and 3 of the ETB allele (a) and those recognizing the Tie2-Cre transgenic construct (b). Products of the ETB allele PCR include the wild-type ETB allele amplicon 'W', the floxed ETB allele amplicon 'F' and recombined ETB allele '0'. The single product of the Tie2-Cre PCR indicates the presence of absence of the Tie2-Cre transgene. Lanes 1-2 therefore describe an EC ETB-deficient mouse (FF/tie2). In these, the Tie2-Cre product is present indicating animals are Tie2-Cre positive. The ETB allele PCR demonstrates the presence of the 'F' floxed ETB amplicon, which is contributed by DNA from non-EC. The '0' recombined ETB amplicon is contributed by DNA from EC and confirms the activity of Cre. Lanes 3-7 describe a floxed, Tie2-Cre negative control animal (FF/--). Here, no product is formed in the Tie2-Cre PCR, and the ETB allele PCR demonstrates only the presence of floxed, non-recombined ETB alleles. EC, endothelial cell. PCR, polymerase chain reaction.

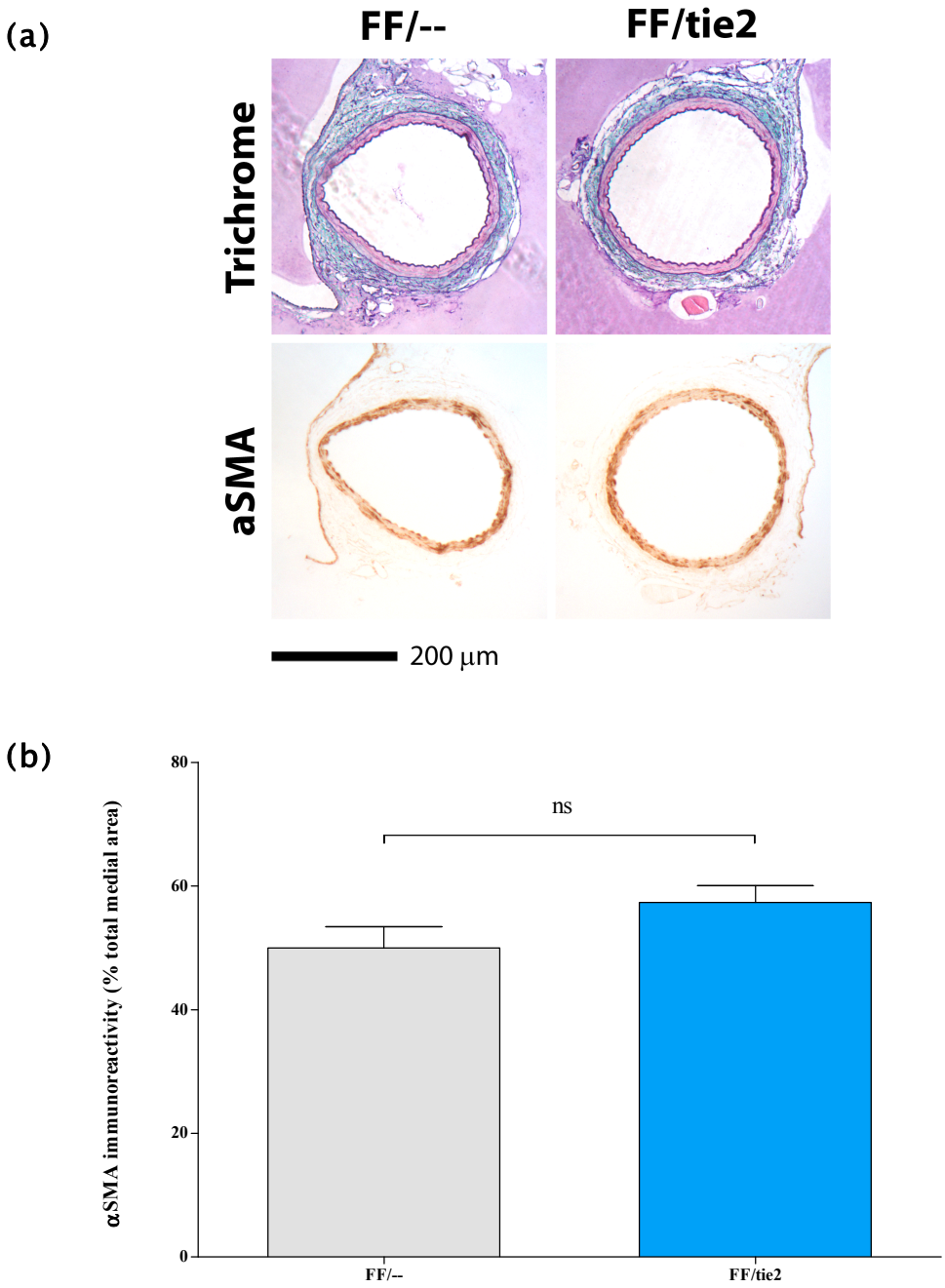


Figure 5-2 Impact of EC ETB-deficiency on femoral artery structure and composition.

Uninjured femoral arteries were perfusion fixed and isolated from FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and examined by United States trichrome staining (a) and αSMA immunohistochemistry (a,b). Immunoreactive area was expressed as a percentage of the total medial area. EC ETB-deficiency had little effect on the outward appearance of uninjured femoral arteries (a) and did not significantly alter the fractional area of αSMA immunoreactivity (b). n=5-8. Data are mean ± SEM. αSMA, α-smooth muscle actin; EC, endothelial cell. ns, $p>0.05$ by Student's unpaired t-test.

small, but significant increase in luminal volume (p=0.035) was observed in lesions from EC-ETB knockout animals at this time point (**Table 5-3**, page 188). OPT measurements were not made of 28 day wire-induced lesions in FF/tie2 or FF/-- mice.

5.3.4.2 *2D morphometry*

Femoral artery wire injury was associated with the development of large, cell- and extracellular matrix-rich neointimal lesions and loss of nuclei from the medial layer (**Figure 5-3**). Lesion size was, in general, considerably larger at 28 days after surgery, than at 14 days, in agreement with previous studies in this laboratory and the literature (Sata *et al.*, 2000).

In agreement with measures derived from OPT images, morphometric analysis, performed on the largest neointimal lesions from each wire-injured femoral artery, indicated no significant effect of genotype on luminal area (day 14: p=0.753; day 28: p=0.593; Figure 5-4), neointimal area (day 14: p=0.348; day 28: p=0.875; Figure 5-4), medial area (day 14: p=0.755; day 28: p=0.788; Table 5-3, page 188), intima/media ratio (day 14: p=0.320; day 28: p=0.870) or stenotic ratio (day 14: p=0.553; day 28: p=0.348).

5.3.4.3 *Lesion composition & proliferation*

Mac2 immunoreactivity was seen in all layers of the vessel wall at both 14 and 28 days post-injury with the greatest densities occurring in the adventitial layer (**Figure 5-3**). Both medial and neointimal macrophage content (fractional Mac2 immunoreactive area) appeared to be substantially greater at 14 days post-injury than 28 days in both genotypes. At neither time point was a significant difference in Mac2 immunoreactivity seen between injured arteries from FF/tie2 and FF/-- mice (**Table 5-3**, page 188) in either the media (day 14: p=0.549; day 28: p=0.710) or neointima (day 14: p=0.744; day 28: p=0.805).

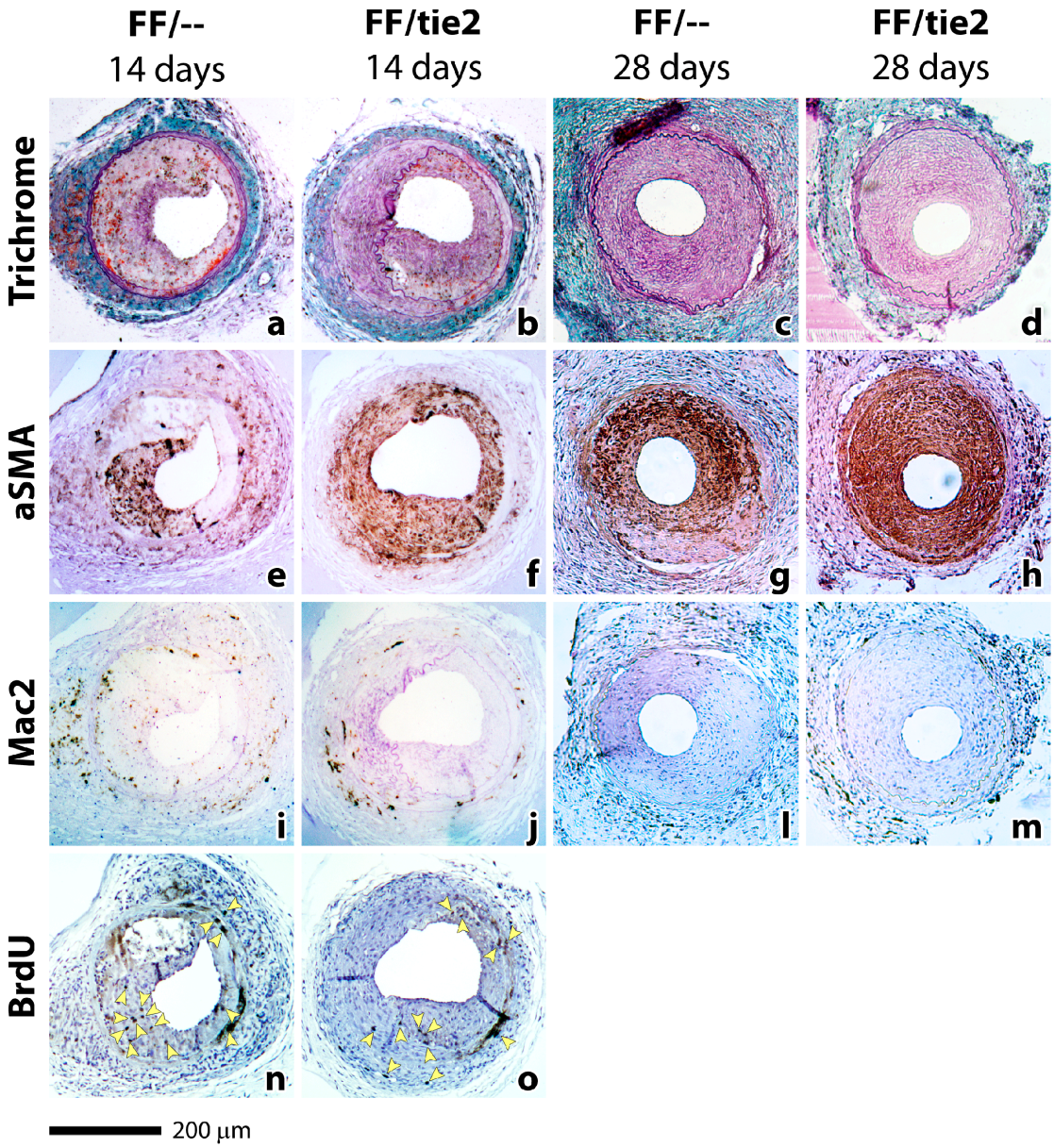


Figure 5-3 Impact of EC ETB-deficiency on femoral artery wire injury-induced lesion size and composition.

Neointima formation was induced by femoral artery wire-injury in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before histological examination. Representative photomicrographs of lesions from both genotypes and time points, stained by United States Trichrome (a-d) and αSMA (e-h), Mac2 (i-m) and BrdU immunohistochemistry (n-o), are shown. Trichrome staining reveals large, elastin (purple) and collagen (blue)-rich lesions that appear smaller at 14 days than 28 days in both genotypes, but similar between genotypes (a-d). αSMA immunoreactivity is strong in the neointima and weaker in the media (e-h). At 14 but not 28 days, neointimal αSMA staining is stronger in FF/tie2 than FF/-- (e-f). At both time points, medial αSMA staining is stronger in FF/tie2 than FF/-- mice (e-h). Punctate Mac2 immunoreactivity present throughout the vascular wall at 14 days in both genotypes (i-j), but is barely present in either at 28 days (l-m). BrdU immunoreactive nuclei (yellow arrowheads) are present in the media and neointima of both genotypes at 14 days (n-o). BrdU staining was not performed on 28 day lesions. αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine; EC, endothelial cell.

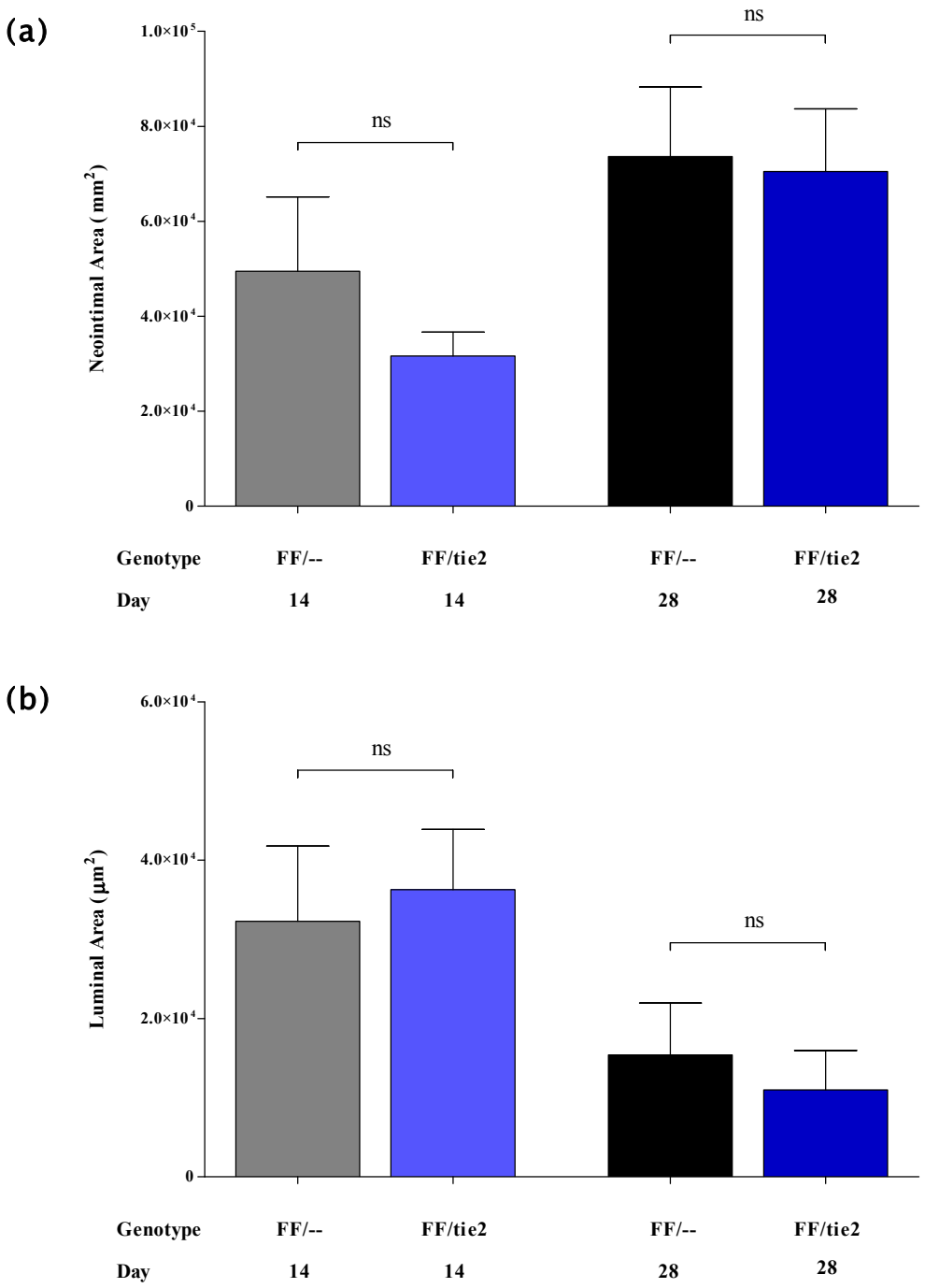


Figure 5-4 Impact of EC ETB-deficiency on neointimal and lumenal area in wire-injured femoral arteries.

Neointima formation was induced by femoral artery wire-injury in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before histological examination. Neointimal (a) and lumenal cross-sectional areas (b) were measured at the point of largest lesion size by morphometry. Neointimal area was not significantly different between genotypes at either time point (a). Similarly, EC ETB-deficiency did not alter lumenal cross-sectional area at 14 or 28 days (b). Although not the basis of statistical testing, in both neointimal areas appeared larger (a), and lumenal areas smaller, at 14 than 28 days (b). n=7-10. Data are mean ± SEM. EC, endothelial cell. ns, $p>0.05$ by Student's unpaired t-test between genotypes at each time point

Immunoreactivity for αSMA was also detectable throughout the vessel wall (**Figure 5-3**). At both 14 and 28 days levels of this antigen in the media were low (as compared to un-injured vessels) and, in the adventitia, staining was variable and most commonly associated with the media-adventitia border and microvasculature. At both 14 ($p=0.003$) and 28 days ($p=0.014$), significantly greater αSMA immunoreactivity was detected in the media of wire-injured femoral arteries from FF/tie2 mice compared to those from FF/-- mice (**Figure 5-5**).

αSMA immunoreactivity was also present in the neointima (**Figure 5-3**). Neointimal lesions from FF/tie2 mice contained fractionally more αSMA immunoreactivity than those from FF/-- mice at 14 ($p=0.012$), but not 28 days ($p=0.295$), post-surgery (**Figure 5-5**). As such, fractional neointimal αSMA immunoreactive area in FF/-- mice appeared to be considerably greater at 28 days than at 14 days post-surgery, whereas in FF/tie2 mice levels were similar at both time-points.

Proliferating nuclei (BrdU immunoreactive) were present in all layers of vessel wall, 14 days post-injury (**Figure 5-3**). The absolute number, however, was not significantly different between genotypes (**Table 5-3**, page 188) in either the media ($p=0.754$) or neointima ($p=0.689$).

5.3.5 *Ligation injury*

5.3.5.1 *3D morphometry*

OPT images, again, revealed clear neointima formation in ligation-injured arteries at 14 and 28 days post-injury which appeared of similar size between time points (**Figure 5-6**), and to those observed in previous studies (chapter 4). Quantification of reconstructed tomograms indicated no effect of EC ETB knockout on neointimal volume (day 14: $p=0.172$; day 28: $p=0.349$), luminal volume (day 14: $p=0.299$; day 28: $p=0.557$) or volumetric stenotic ratio (day 14: $p=0.473$; day 28: $p=0.969$) at either examined time point (**Table 5-4**, page 189).

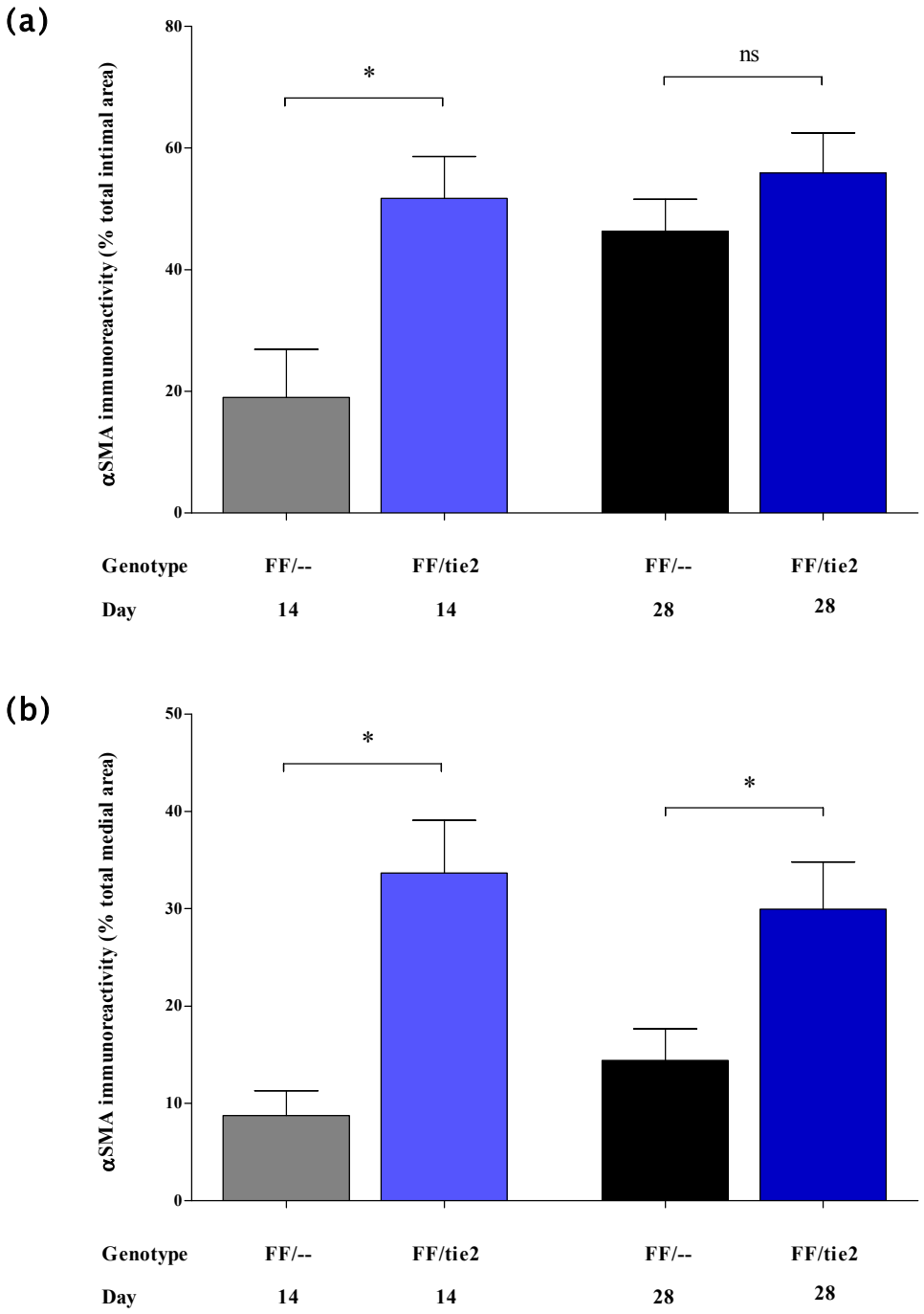


Figure 5-5 Impact of EC ETB-deficiency on neointimal and medial αSMA immunoreactivity in wire-injured femoral arteries.

Neointima formation was induced by femoral artery wire-injury in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before examination by αSMA immunohistochemistry. Immunoreactive area was expressed as % of the total area of the appropriate layer of the vascular wall. At 14 but not 28 days, EC ETB-deficiency was associated with a significant increase in fractional αSMA immunoreactive area. At both 14 and 28 days, medial αSMA stained area was greater in lesions from FF/tie2 mice than in those from control FF/-- animals. n=6-10. Data are mean ± SEM. αSMA, α-smooth muscle actin; EC, endothelial cell. ns, $p>0.05$; *, $p<0.05$ by Student's unpaired t-test between genotypes at each time point.

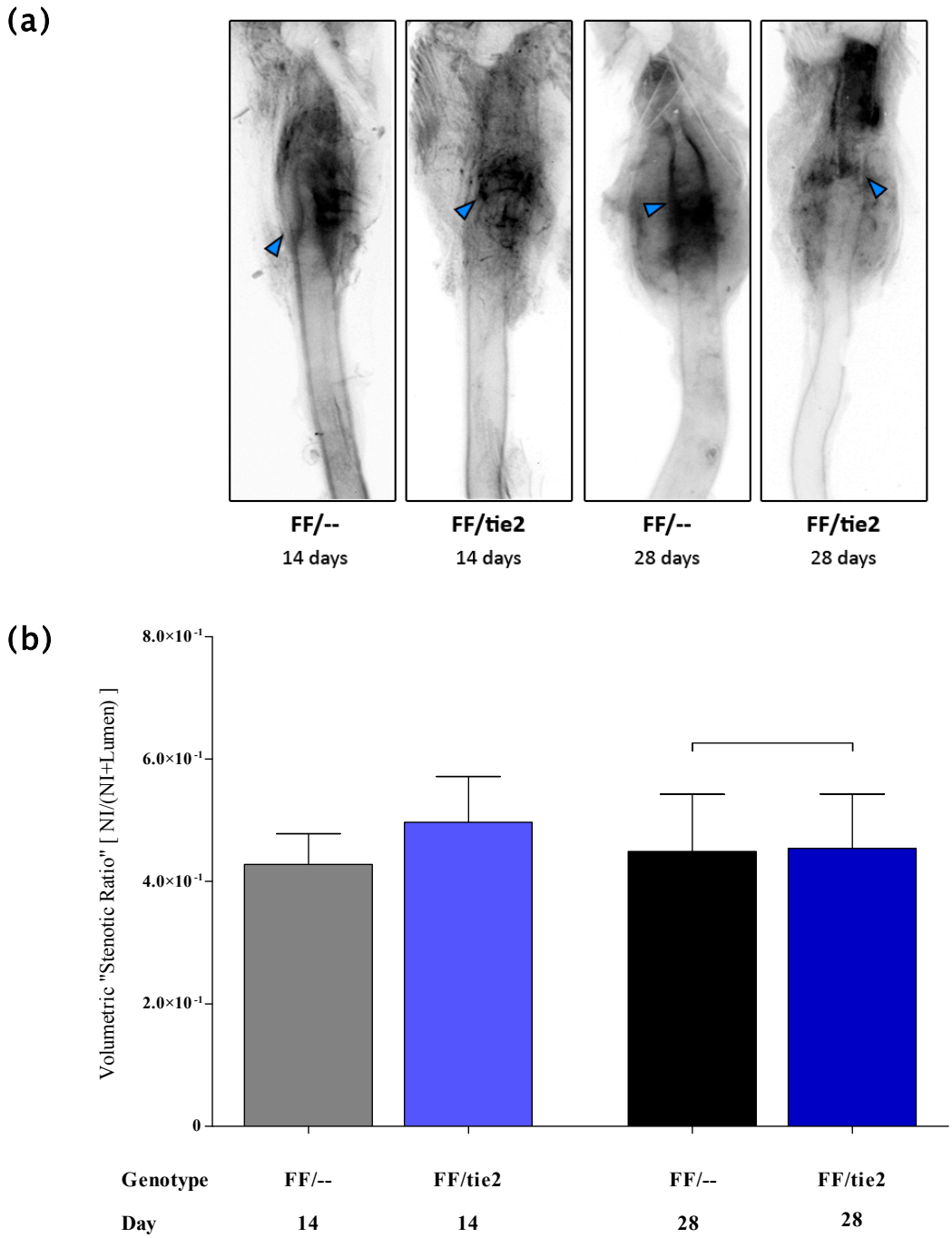


Figure 5-6 Impact of EC ETB-deficiency on complete femoral artery ligation-induced neointimal lesion volume, as assessed by optical projection tomography.

Neointima formation was induced by complete femoral artery ligation-injury in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before examination by optical projection tomography. Lesion formation and adventitial inflammation were clearly visible in raw projection images (a, arrowheads). Lesion volumes were recorded from reconstructed tomograms and expressed as a fraction of vessel occupied by lesion. Fractional lesion volume was not altered by EC ETB-deficiency. Lesion volumes also appeared similar between 14 and 28 days points in both genotypes. n=5-8. Data are mean ± SEM. NI, neointimal area; EC, endothelial cell. ns, $p>0.05$ by Student's unpaired t-test between genotypes at each time point.

5.3.5.2 *2D morphometry*

As previously discussed (chapters 3 and 4), complete ligation of the femoral artery across the femoropopliteal bifurcation resulted in the formation of localised neointimal lesions (**Figure 5-7**). These appeared somewhat smaller than those elicited by wire-injury.

Neointimal cross-sectional area was similar at 14 (p=0.228) and 28 (p=0.429) days post-ligation in both genotypes (**Figure 5-8**). EC ETB genotype was also not associated with significant changes in luminal area (day 14: p=0.286; day 28: p=0.883; **Figure 5-8**), medial area (day 14: p=0.947; day 28: p=0.487), intima/media ratio (day 14: p=0.233; day 28: p=0.829) or stenotic ratio (day 14: p=0.209; day 28: p=0.651) at 14 or 28 days post-ligation (**Table 5-4**, page 189).

5.3.5.3 *Lesion composition & proliferation*

In ligation-injured vessels, distribution of Mac2 immunoreactivity was broadly similar to that seen in wire-injured vessels at 28 days with staining present in the adventitia, media and, more weakly, in the neointima (**Figure 5-7**). Lesions induced by this method had obvious differences in the pattern of αSMA immunoreactivity to those induced by wire-injury. Here, staining was intense throughout both the media and neointima and was present at similar levels between 14 and 28 days (**Figure 5-7**). Adventitial αSMA immunoreactivity was variably present at 14 days but rarely at 28 days. BrdU immunoreactivity was common at 14 days, and in all layers of the vascular wall but again, appeared less frequent at 28 days. (**Figure 5-7**).

Neointimal macrophage content (Mac2 immunoreactivity; day 14: p=0.306; day 28: p=0.527), smooth muscle-like cell content (αSMA immunoreactivity; day 14: p=0.524; day 28: p=0.255) and proliferating cell count (BrdU immunoreactivity; day 14: p=0.703; day 28: p=0.833) were not significantly different between ligation-induced lesions in FF/-- and FF/tie2 mice at 14 or 28 days post-injury (**Table 5-4**, page 189). Similarly, in the media, Mac2 (day 14: p=0.796; day 28: p=0.720), αSMA

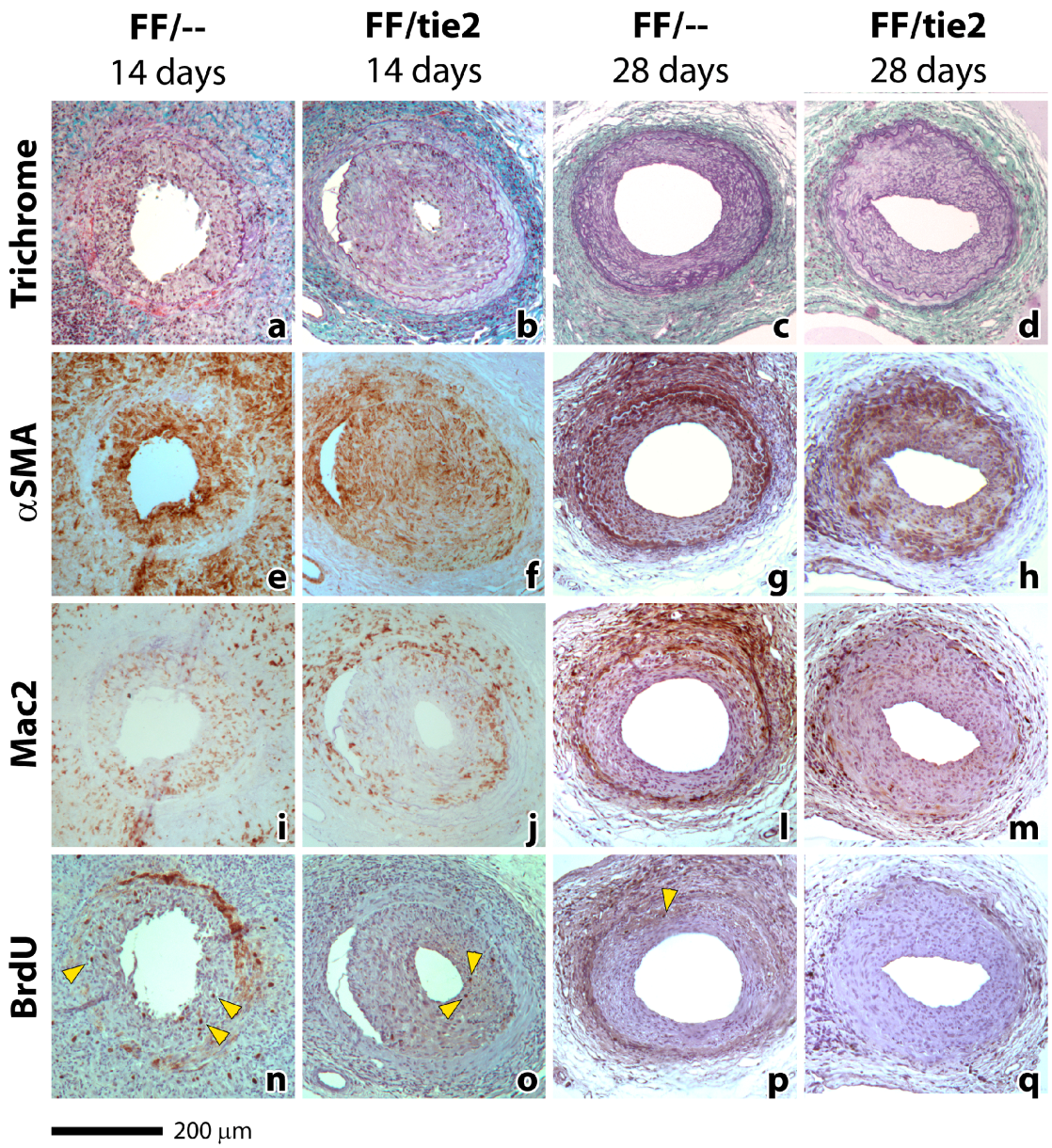


Figure 5-7 Impact of EC ETB-deficiency on complete femoral artery ligation injury-induced lesion size and composition.

Neointima formation was induced by complete femoral artery ligation-injury in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before histological examination. Representative photomicrographs of lesions from both genotypes and time points, stained by United States Trichrome (a-d) and αSMA (e-h), Mac2 (i-m) and BrdU immunohistochemistry (n-q), are shown. Trichrome staining reveals large, elastin (purple) and collagen (blue)-rich lesions that are similar in size in all genotypes and time points (a-d). αSMA immunoreactivity is strong in the neointima and media (e-h). In some vessels, αSMA staining is also associated with the adventitia at 14 but not 28 days. Mac2 immunoreactivity present throughout the vascular wall at 14 and 28 days in both genotypes (i-m). BrdU immunoreactive nuclei (yellow arrowheads) were common in lesions from both FF/tie2 and FF/-- mice at 14 days in the neointima, media and adventitia (n-o), but were rarely present at 28 days (p-q). αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine; EC, endothelial cell.

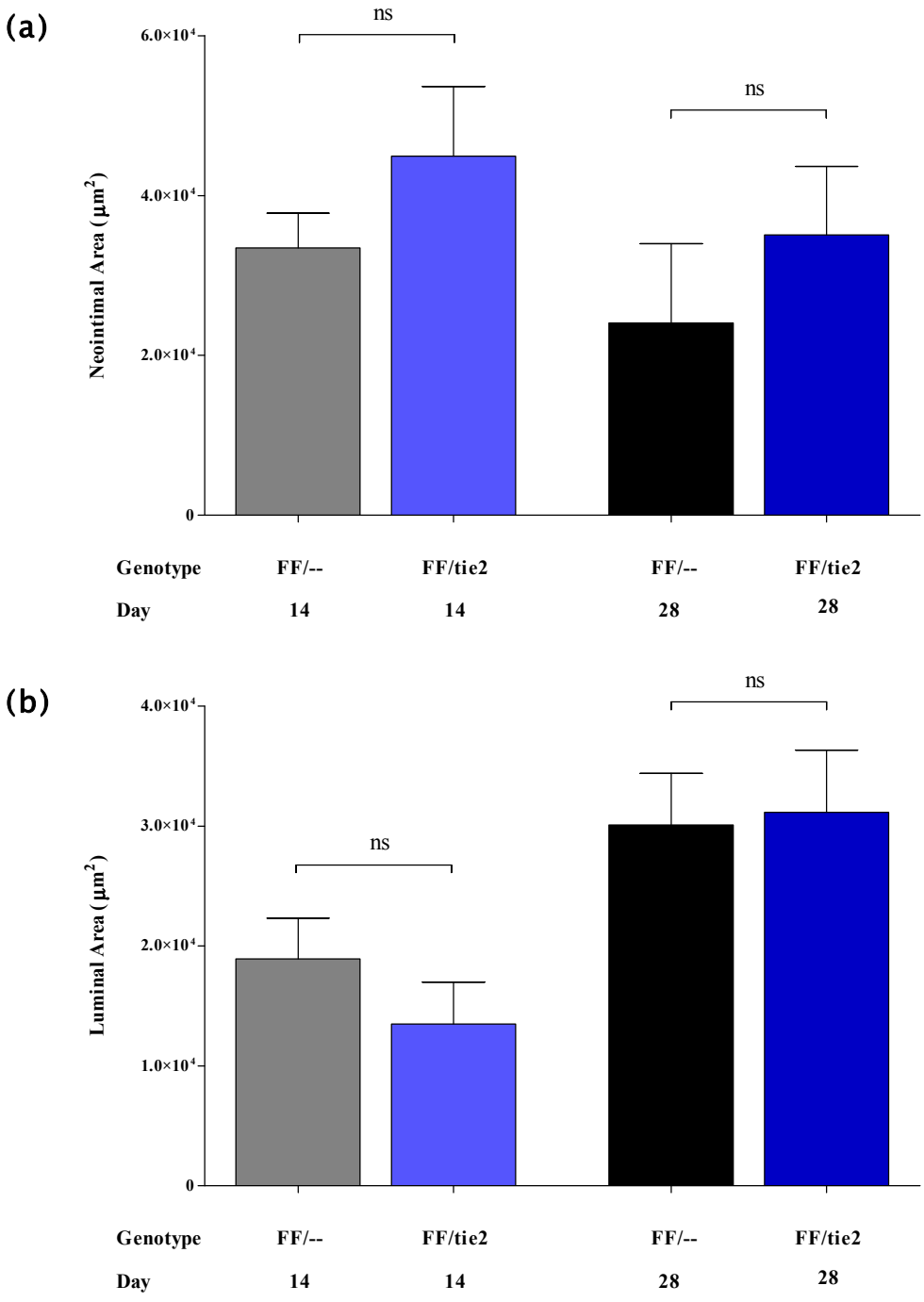


Figure 5-8 Impact of EC ETB-deficiency on neointimal and lumenal area in complete ligation-injured femoral arteries.

Neointima formation was induced by complete femoral artery ligation0-injury in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before histological examination. Neointimal (a) and lumenal cross-sectional areas (b) were measured at the point of largest lesion size by morphometry. Neointimal area was not significantly different between genotypes at either time point (a). Similarly, EC ETB-deficiency did not alter lumenal cross-sectional area at 14 or 28 days (b). Neointimal areas appeared similar in size in lesions from both genotypes at 14 and 28 days. n=7-10. Data are mean ± SEM. EC, endothelial cell. ns, $p>0.05$ by Student's unpaired t-test between genotypes at each time point.

(day 14: p=0.594; day 28: p=0.549) and BrdU immunoreactivity (day 14: p=0.191; day28: p=0.725) did not differ between genotypes (**Table 5-4**, page 189).

5.3.6 *Vasomotor function*

5.3.6.1 *Vasoconstrictor responses to phenylephrine and KPSS*

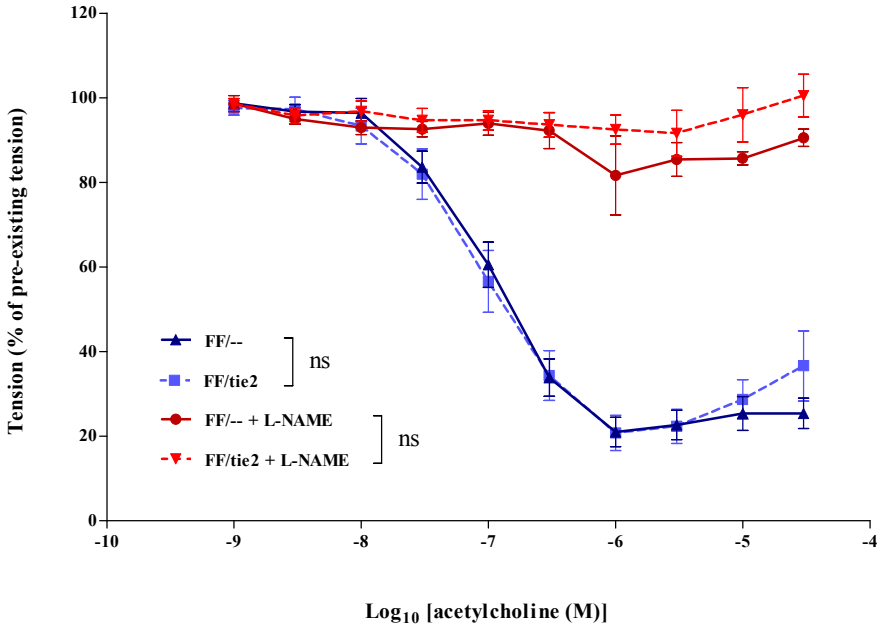
In femoral arteries isolated from mice of all ages and genotypes tested, the α_1-adrenoceptor agonist phenylephrine stimulated concentration-dependent contraction. The response to this agent was significantly increased in vessels from young (p=0.014) but not aged EC-ETB knockout animals (**Table 5-5**, page 190). In the aorta of FF/tie2 mice, a small but significant decrease in the response to phenylephrine was observed (**Table 5-6**, page 191).

Increasing K^+ concentrations in the solution bathing femoral arteries also resulted in constriction of all tested vessels. The magnitude and sensitivity of this response was not altered by EC ETB genotype in young or aged mice (**Table 5-5**, page 190).

5.3.6.2 *Vasodilator responses to acetylcholine and sodium nitroprusside*

Acetylcholine stimulated concentration-dependent relaxation of phenylephrine pre-contracted femoral artery rings and this action was almost abolished by pre-incubation with an inhibitor of NO synthases, L-NAME (**Figure 5-9**). The maximum relaxation elicited appeared to be reduced by ageing, but in neither young nor aged animals was a difference in any parameter of this response seen between FF/tie2 and FF/-- mouse vessels. In isolated aortas, a significant difference between genotypes in the response to acetylcholine was present (**Figure 5-10**). This was only apparent at high concentrations where, in FF/tie2 mouse vessels, a contractile phase of the response occurred at lower acetylcholine concentrations than in aortas from FF/-- mice. As a consequence of this bi-phasic response, it was not possible to derive summary data from this response. In the presence of L-NAME, the relaxant effect of acetylcholine in aortic rings was abolished, and the contractile phase occurring at high concentrations was still present. No difference in these responses was noted between genotypes.

(a) Young (2-4 month old)



(b) Aged (7-10 month old)

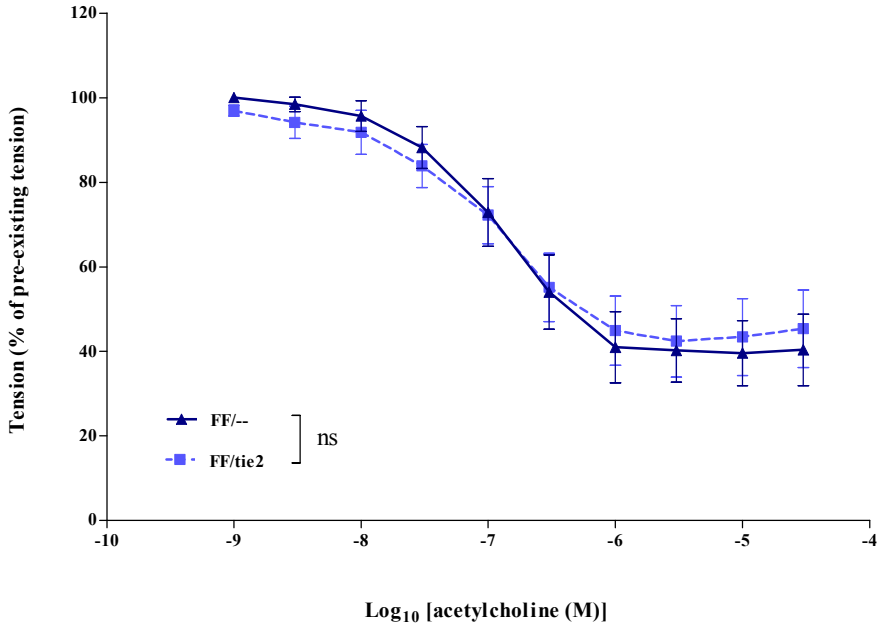


Figure 5-9 Impact of EC ETB-deficiency on the vasodilator response to acetylcholine in isolated femoral arteries from young and aged mice.

Femoral artery rings from young (2-4 month old) and aged (7-10 month old), EC ETB-deficient (FF/tie2) and littermate control (FF/--) mice were examined by wire myography. Acetylcholine elicited relaxation of pre-contracted vessels that was not different between genotypes in either young (a) or aged groups (b). Pre-incubation with the nitric oxide synthase inhibitor, L-NAME ($2x10^{-4}$M) abolished the response to acetylcholine in vessels from young mice of both genotypes (a). n=6-19. Data are mean ± SEM. EC, endothelial cell. ns, $p>0.05$ by two-way ANOVA between genotypes, within each age/treatment group.

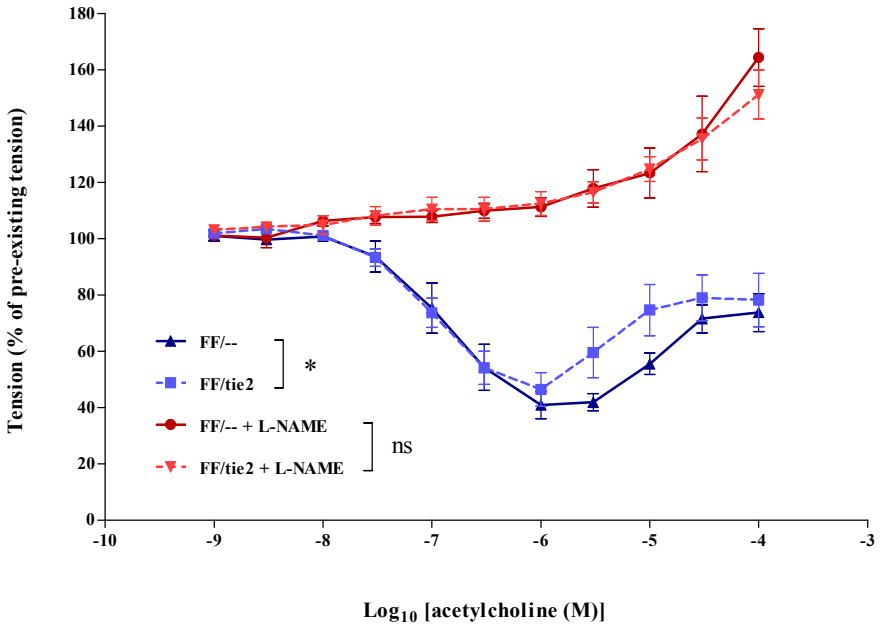


Figure 5-10 Impact of EC ETB-deficiency on the vasomotor response to acetylcholine in isolated aortas.

Aortic rings from young (2-4 month old) EC ETB-deficient (FF/tie2) and littermate control (FF/--) mice were examined by wire myography. The response to acetylcholine appeared bi-phasic comprising relaxation at low concentration, and re-contraction at high concentration. In FF/tie2 mouse aortas the contractile phase of the response occurred at lower concentration than in aortas from FF/-- mice. Pre-incubation with the nitric oxide synthase inhibitor L-NAME ($2x10^{-4}$M) abolished the relaxant but not contractile phase in both genotypes. The response to acetylcholine in the presence of L-NAME was not different in between FF/tie2 and FF/-- mouse aortas. n = 5-6. Data are mean ± SEM. EC, endothelial cell. ns, $p>0.05$; *, $p<0.05$ by two-way ANOVA between genotypes, within each treatment group.

Pre-constricted vessels from FF/tie2 and FF/-- mice relaxed in response to increasing concentrations of the NO donor drug sodium nitroprusside. Neither age nor genotype altered any parameters of this response (**Table 5-5**, page 190).

5.3.6.3 *Vasomotor responses to ET agonists*

Endothelin-1 elicited vasoconstriction of femoral artery rings from mice of all ages and genotypes (**Figure 5-11**). This response was not different between vessels taken from FF/tie2 mice and FF/-- mice, in either young or aged groups. In the presence of BQ123, a selective ETA receptor antagonist, a parallel rightward shift in the concentration-response curve was observed, the magnitude of which was not different between any experimental groups. In femoral arteries from young FF/-- mice, pre-incubation with A192621, a selective ETB receptor antagonist, did not alter the sensitivity or maximum response to ET-1 (**Figure 5-11**). In contrast, pre-treatment with this antagonist was able to completely abolish the contractile response of isolated rings of mouse trachea to sarafotoxin S6c (data not shown).

Sarafotoxin 6c (10^{-7}M) elicited a small but definite contraction in isolated femoral arteries pre-treated with L-NAME (**Figure 5-12**), the magnitude of which was not different (p=0.402) between FF/-- (5.72 ± 1.62 %) and FF/tie2 mice (3.67 ± 1.70 %). In phenylephrine pre-contracted femoral arteries, some response was also apparent but its presence, size and direction proved highly inconsistent between experiments (**Figure 5-12**).

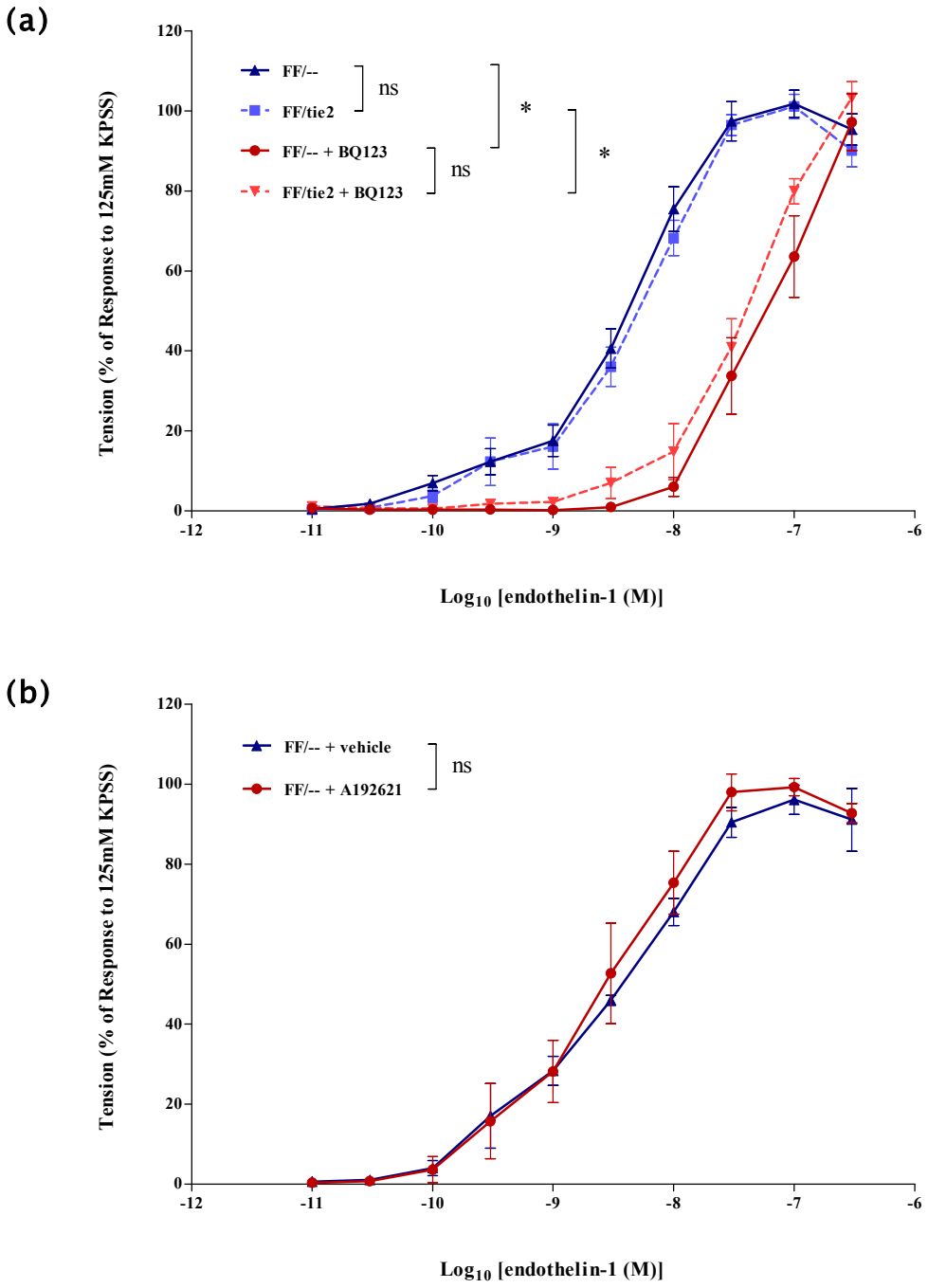


Figure 5-11 Impact of EC ETB-deficiency and acute endothelin receptor blockade on the vasoconstrictor response to ET-1 in isolated femoral arteries.

Femoral artery rings from young (2-4 month old) EC ETB-deficient (FF/tie2) and littermate control (FF/--) mice were examined by wire myography. In all vessels, ET-1 potently stimulated contraction. This response did not differ between vessels from EC ETB-deficient and control mice (a). Pre-incubation with the selective ETA antagonist, BQ123 (10^{-6}M) resulted in a rightward shift in the concentration-response curve to ET-1 of similar extent in both genotypes (a). The response to ET-1 in FF/-- femoral arteries was not altered by pre-incubation with the selective ETB antagonist A192621 (b; 10^{-7}M). KPSS, high potassium physiological salt solution, n = 3-9. Data are mean ± SEM. EC, endothelial cell. ns, $p>0.05$; *, $p<0.05$ by two-way ANOVA between genotypes (a) or treatments (b).

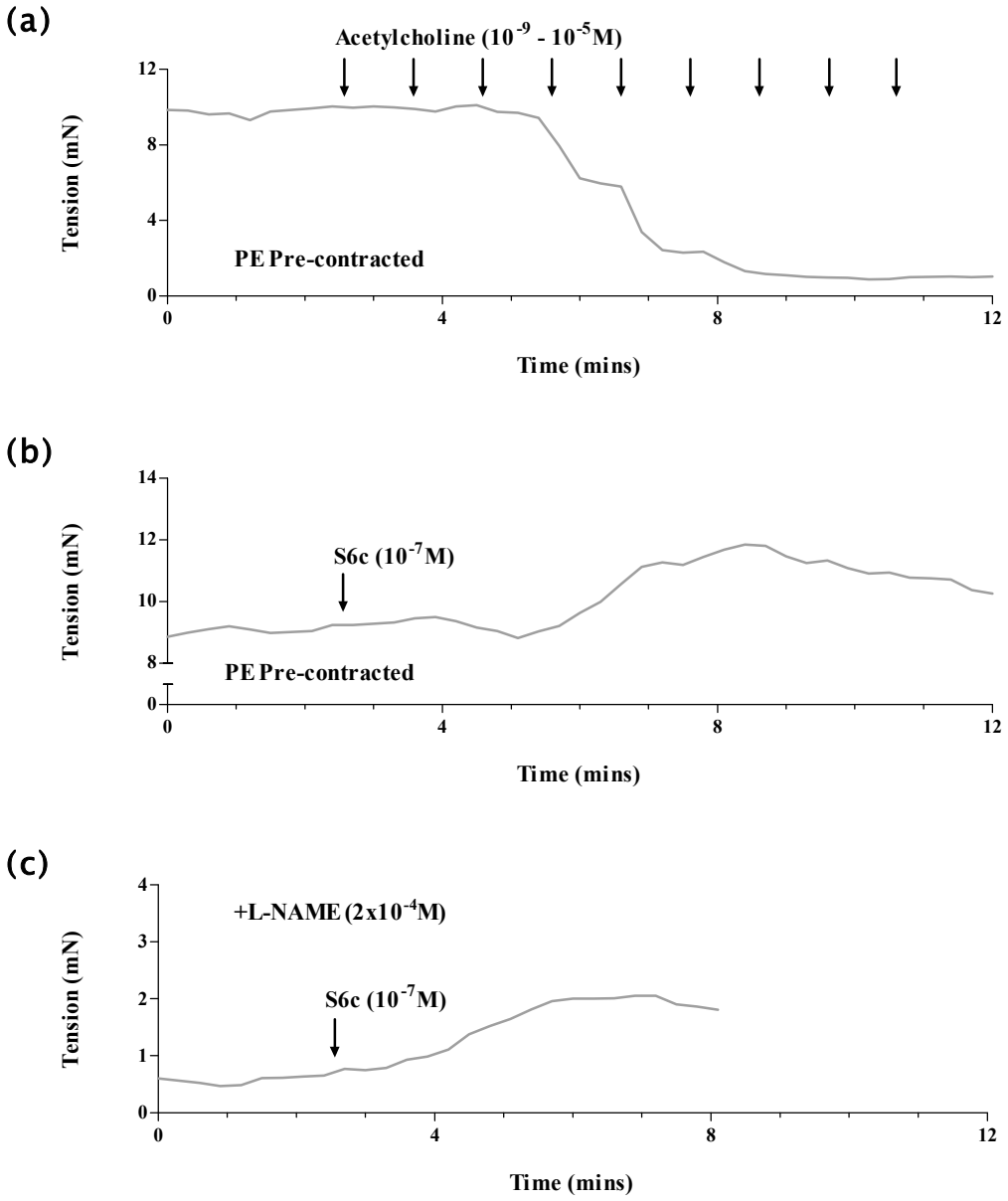


Figure 5-12 Vasomotor responses of isolated femoral arteries to sarafotoxin S6c.

Femoral artery rings from young (2-4 month old) control (FF/--) mice were examined by wire myography. Representative traces of tension changes in femoral artery rings exposed to acetylcholine (a) or to the ETB agonist sarafotoxin S6c are shown (b,c). Stepwise addition of acetylcholine (10^{-9}-10^{-5}M) elicits strong concentration-dependent relaxation of phenylephrine pre-constricted femoral arteries (a). Application of S6c (10^{-7}M) to phenylephrine pre-constricted femoral arteries elicited extremely variable responses possibly comprising dilator and constrictor components (b). In un-constricted vessels, pre-treated with the nitric oxide synthase inhibitor L-NAME ($2x10^{-4}$M), application of S6c (10^{-7}M) reliably elicited a small but definite contraction (c). Traces are representative of at least 3 experiments. S6c, sarafotoxin S6c.

Genotype	**FF/--**	**FF/tie2**	**FF/--**	**FF/tie2**
Time-point of relevant study	14 days	14 days	28 days	28 days
(a) Wire Injury Studies				
Body weight at study start (**g**)	29.11 ± 1.38	30.60 ± 0.67	27.50 ± 1.30	29.10 ± 0.80
Body weight at study end (**g**)	28.76 ± 1.29	29.73 ± 0.55	28.20 ± 1.10	29.20 ± 2.00
Body weight change (**g**)	-0.36 ± 0.28	-0.87 ± 0.53	0.70 ± 0.60	0.20 ± 2.40
(b) Ligation Injury Studies				
Body weight at study start (**g**)	as (a)	as (a)	30.83 ± 1.77	30.11 ± 0.62
Body weight at study end (**g**)	as (a)	as (a)	Not recorded	Not recorded
Body weight change (**g**)	as (a)	as (a)	Not recorded	Not recorded

Genotype	**FF/--**	**FF/tie2**	**FF/--**	**FF/tie2**
Age Group	Young	Young	Aged	Aged
(c) Vascular Function Studies				
Age Range **(weeks)**	11-20	13-19	41-47	43-47
Mean Age **(weeks)**	15.7 ± 0.4	15.1 ± 0.6	44.6 ± 0.7	45.4 ± 0.4
Body weight (**g**)	31.1 ± 1.3	31.2 ± 0.9	45.8 ± 2.6	49.0 ± 4.3
Heart weight (**mg**)	202.0 ± 8.1	209.6 ± 10.2	222.7 ± 15.6	229.3 ± 14.7
Liver weight (**mg**)	1906 ± 68.0	1782 ± 32.0	2287 ± 119.0	2204 ± 221.3
Total kidney weight (**mg**)	614.1 ± 38.3	567.0 ± 25.0	675.5 ± 41.9	728.3 ± 41.5

Table 5-1 Impact of EC-specific ETB deficiency on body and organ weights.

EC ETB-deficient mice (FF/tie2) and their littermate controls (FF/--) were weighed at beginning and end of femoral artery injury and vascular function studies, and in the latter, organs were removed and weighed. Pre- and post-operative body weights were not different for mice undergoing any of the vascular injury studies presented in this chapter. Similarly, neither body weights nor the weight of major organs (liver, kidneys, heart) of mice used for vascular function studies differed between genotypes, in either young or aged groups. For organ weights, n = 4-6. EC, endothelial cell.

Genotype	FF/--	FF/tie2
(a) 2D Morphometry (Histology)		
n	5	8
Luminal area (**x10^{3}µm^2**)	47.65 ± 5.25	47.95 ± 1.56
Medial area (**x10^{3}µm^2**)	14.06 ± 0.87	16.71 ± 1.25
Lumen/Media ratio	3.39 ± 0.08	3.20 ± 0.20
(b) Composition (Immunohistochemistry)		
n	5	8
Medial αSMA IR (**%**)	50.0 ± 3.4	57.3 ± 2.7

Table 5-2 Impact of EC ETB-deficiency on femoral artery structure and medial composition.

Uninjured femoral arteries were perfusion fixed and isolated from FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and examined by United States trichrome staining (a) and αSMA immunohistochemistry (b). No differences in histological measures of morphology (a) or of medial αSMA immunoreactivity were apparent between genotypes. Data are mean ± SEM. αSMA, α-smooth muscle actin; EC, endothelial cell.

Genotype	**FF/--**	**FF/tie2**	**FF/--**	**FF/tie2**
Time-point of relevant study	14 days	14 days	28 days	28 days
(a) 3D Morphometry (OPT)				
n	10	10		
Intimal volume (**x10^{7}µm^3**)	6.34 ± 0.18	5.18 ± 0.72	-	-
Luminal volume (**x10^{7}µm^3**)	**20.34 ± 1.92**	**25.40 ± 1.10 ***	-	-
Volumetric stenotic ratio	0.23 ± 0.05	0.17 ± 0.02	-	-
(b) 2D Morphometry (Histology)				
n	10	9	7	8
Luminal area (**x10^{3}µm^2**)	32.28 ± 0.95	36.26 ± 0.76	15.42 ± 0.65	10.97 ± 0.50
Neointimal area (**x10^{3}µm^2**)	49.46 ± 15.74	31.66 ± 0.50	73.66 ± 14.65	70.49 ± 13.19
Medial area (**x10^{3}µm^2**)	21.86 ± 0.23	22.98 ± 0.27	25.23 ± 0.55	27.82 ± 0.74
Intima/Media ratio	2.93 ± 1.14	1.58 ± 0.33	3.22 ± 0.73	3.09 ± 0.40
Stenotic ratio	0.57 ± 0.12	0.48 ± 0.10	0.81 ± 0.06	0.88 ± 0.04
(c) Composition & Proliferation (Immunohistochemistry)				
n	10	9	7	7
Intimal αSMA IR (**%**)	**19.0 ± 8.0**	**51.7 ± 6.9 ***	46.3 ± 5.2	55.9 ± 6.6
Intimal Mac2 IR (**%**)	3.8 ± 0.8	4.5 ± 0.9	1.2 ± 1.0	1.1 ± 0.8
Intimal BrdU IR (**count**)	9.7 ± 1.8	8.4 ± 1.7	-	-
Medial αSMA IR (**%**)	**8.8 ± 2.6**	**33.7 ± 5.4 ** **	**14.4 ± 3.2**	**29.9 ± 4.9 ***
Medial Mac2 IR (**%**)	4.6 ± 0.8	5.4 ± 1.1	0.7 ± 0.3	0.9 ± 0.3
Medial BrdU IR (**count**)	7.6 ± 2.6	6.8 ± 1.6	-	-

Table 5-3 Impact of EC ETB-deficiency on the size and composition of femoral artery wire injury-induced neointimal lesions.

Neointima formation was induced by femoral artery wire-injury in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before examination by optical projection tomography (a), histology (b) and immunohistochemistry (c). Optical projection tomography indicated no difference in lesion volume between genotypes at 14 days, but vessels from FF/tie2 mice showed a significant increase in luminal volume (a). No histological measures were altered by genotype at either 14 or 28 days post-injury (b). Intimal αSMA immunoreactivity was significantly greater in lesions from FF/tie2 mice as compared to those from FF/-- mice at 14 but not 28 days post-injury. In the media, αSMA staining was increased by EC ETB-deficiency at both 14 and 28 days post-injury. No other compositional changes were noted between genotypes. Data are mean ± SEM. αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine; EC, endothelial cell. *, $p<0.05$; **, $p<0.01$ by Student's unpaired t-test between genotypes, at each time point.

Genotype	**FF/--**	**FF/tie2**	**FF/--**	**FF/tie2**
Time-point of relevant study	14 days	14 days	28 days	28 days
(a) 3D Morphometry (OPT)				
n	8	9	5	7
Intimal volume (**x10^{7}µm^3**)	5.41 ± 0.70	8.96 ± 2.25	4.48 ± 0.88	6.55 ± 1.65
Luminal volume (**x10^{7}µm^3**)	6.93 ± 0.59	8.05 ± 0.81	6.08 ± 1.59	7.16 ± 1.00
Volumetric stenotic ratio	0.43 ± 0.05	0.50 ± 0.07	0.45 ± 0.09	0.45 ± 0.89
(b) 2D Morphometry (Histology)				
n	10	8	7	9
Luminal area (**x10^{3}µm^2**)	18.92 ± 0.34	13.49 ± 0.35	30.09 ± 0.43	31.14 ± 0.52
Neointimal area (**x10^{3}µm^2**)	33.46 ± 0.43	44.92 ± 0.87	24.08 ± 0.99	35.07 ± 0.86
Medial area (**x10^{3}µm^2**)	29.10 ± 0.29	29.40 ± 0.35	19.22 ± 0.21	21.10 ± 0.16
Intima/Media ratio	1.16 ± 0.14	1.48 ± 0.23	1.57 ± 0.52	1.72 ± 0.46
Stenotic ratio	0.62 ± 0.06	0.75 ± 0.08	0.41 ± 0.10	0.48 ± 0.09
(c) Composition & Proliferation (Immunohistochemistry)				
n	10	8	7	9
Intimal αSMA IR (**%**)	45.0 ± 6.6	37.9 ± 9.0	41.3 ± 7.6	51.2 ± 4.5
Intimal Mac2 IR (**%**)	7.0 ± 2.7	3.6 ± 1.3	5.0 ± 2.0	3.5 ± 1.3
Intimal BrdU IR (**count**)	7.1 ± 2.1	6.1 ± 1.0	3.6 ± 2.2	3.0 ± 1.7
Medial αSMA IR (**%**)	43.5 ± 6.3	38.5 ± 6.7	48.6 ± 8.2	43.1 ± 4.7
Medial Mac2 IR (**%**)	3.2 ± 0.8	3.7 ± 1.9	4.7 ± 2.2	5.8 ± 2.1
Medial BrdU IR (**count**)	3.6 ± 1.3	1.5 ± 0.3	1.1 ± 0.6	1.6 ± 0.9

Table 5-4 Impact of EC ETB-deficiency on the size and composition of complete femoral artery ligation-induced neointimal lesions.

Neointima formation was induced by complete ligation of the femoral artery in FF/tie2 (EC ETB-deficient) and FF/-- (littermate control) mice and lesions allowed to develop for 14 or 28 days before examination by optical projection tomography (a), histology (b) and immunohistochemistry (c). Both optical projection tomographic (a) and histological analysis (b) indicated no effect of genotype on vascular morphology at either 14 or 28 days post-injury. Similarly, no compositional differences were noted between lesions from FF/tie2 and FF/-- mice (c). Data are mean ± SEM. αSMA, α-smooth muscle actin; BrdU, 5-bromo-2-deoxyuridine; EC, endothelial cell.

Genotype		**FF/--**	**FF/tie2**	**FF/--**	**FF/tie2**
Age group		Young	Young	Aged	Aged
PE	n	19	12	8	8
	pD_2	6.00 ± 0.08	5.86 ± 0.12	6.07 ± 0.06	5.96 ± 0.08
	E_{max} (%)	85.4 ± 2.5	94.2 ± 7.2	97.1 ± 5.2	97.4 ± 1.4
	E_{max} ($mN.mm^{-1}$)	2.31 ± 0.21	2.17 ± 0.27	2.44 ± 0.27	2.54 ± 0.43
KPSS	n	6	4	6	6
	pD_2	1.63 ± 0.03	1.63 ± 0.03	1.59 ± 0.03	1.59 ± 0.01
	E_{max} (%)	103.2 ± 4.2	97.7 ± 3.3	111.3 ± 5.4	110.2 ± 2.7
	E_{max} ($mN.mm^{-1}$)	2.36 ± 0.39	3.13 ± 0.46	2.38 ± 0.30	2.28 ± 0.26
ACh	n	19	10	8	6
	$-log(EC_{50})$	7.03 ± 0.08	7.19 ± 0.10	6.92 ± 0.15	7.41 ± 0.44
	E_{max} (%)	77.4 ± 3.3	79.4 ± 3.3	54.4 ± 8.0	59.9 ± 9.5
SNP	n	6	4	6	6
	$-log(EC_{50})$	8.00 ± 0.10	7.88 ± 0.08	7.96 ± 0.08	7.95 ± 0.05
	E_{max} (%)	97.5 ± 0.8	97.5 ± 0.6	95.2 ± 2.8	97.2 ± 1.0
ET-1	n	9	6	6	6
	pD_2	8.26 ± 0.10	8.32 ± 0.07	8.42 ± 0.07	8.34 ± 0.10
	E_{max} (%)	103.2 ± 4.2	101.9 ± 5.8	106.4 ± 5.6	105.8 ± 6.5
	E_{max} ($mN.mm^{-1}$)	2.42 ± 0.18	2.99 ± 0.50	**3.17 ± 0.31**	**1.68 ± 0.21 ****
ET-1 +BQ123	n	6	4	6	6
	pD_2	7.20 ± 0.14	7.40 ± 0.12	7.16 ± 0.15	7.28 ± 0.12
	E_{max} (%)	110.1 ± 6.9	97.7 ± 13.6	126.8 ± 11.5	115.9 ± 5.6
	E_{max} ($mN.mm^{-1}$)	1.61 ± 0.68	2.97 ± 0.36	1.55 ± 0.36	2.87 ± 0.52
ET-1 +vehicle for A192621	n	3	-	-	-
	pD_2	8.73 ± 0.09	-	-	-
	E_{max} (%)	112.9 ± 10.4	-	-	-
	E_{max} ($mN.mm^{-1}$)	3.11 ± 0.28	-	-	-
ET-1 +A192621	n	3	-	-	-
	pD_2	8.29 ± 0.14	-	-	-
	E_{max} (%)	103.6 ± 3.4	-	-	-
	E_{max} ($mN.mm^{-1}$)	3.46 ± 0.28	-	-	-

Table 5-5 Impact of EC ETB-deficiency on femoral artery vasomotor function.

Femoral artery rings from young (2-4 month old) and aged (7-10 month old), EC ETB-deficient (FF/tie2) and littermate control (FF/--) mice were examined by wire myography. No parameters of any measured response differed between FF/tie2 and FF/-- mouse femoral arteries except the absolute E_{max} response to ET-1 in aged vessels, which was significantly reduced in vessels from FF/tie2 mice. Data are mean ± SEM PE, phenylephrine; KPSS, high potassium physiological salt solution; ACh, acetylcholine; SNP, sodium nitroprusside; ET-1, endothelin-1; EC, endothelial cell. **, $p<0.01$ by Student's unpaired t-test between genotypes, within age group.

Genotype		FF/--	FF/tie2
PE	n	6	6
	pD_2	6.50 ± 0.11	6.46 ± 0.05
	E_{max} (%)	**131.7 ± 7.7**	**105.5 ± 5.0 ***
	E_{max} ($mN.mm^{-1}$)	1.68 ± 0.16	1.23 ± 0.19

Table 5-6 Impact of EC ETB-deficiency on aortic vasomotor function.

Aortic rings from young (2-4 month old) EC ETB-deficient (FF/tie2) and littermate control (FF/--) mice were examined by wire myography. The relative Emax response, but neither pD2 not absolute Emax response to phenylephrine was significantly impaired in aortas from FF/tie2 mice as compared to FF/-- controls. Data are mean ± SEM PE, phenylephrine; EC, endothelial cell. *, $p<0.05$ by Student's unpaired t-test between genotypes, within age group.

5.4 DISCUSSION

The experiments described in this chapter were conducted to determine the effects of selective deficiency of ETB from endothelial cells on neointimal lesion formation following acute femoral artery injury. The results suggest that ETB receptors expressed by EC contribute little to the response to vascular injury, and this is consistent with a similar lack of effect of EC ETB-deficiency on endothelium-dependent vasodilatation and other vasomotor function in the femoral artery. These results could reflect the methodological approaches used. When compared with the impact of non-specific ETB blockade/deletion previously observed (Chapter 4 and Murakoshi *et al* 2002), however, they may suggest that ETB expressed in cells other than the vascular endothelium act to moderate neointimal proliferation.

5.4.1 *EC ETB deficiency does not alter endothelium-dependent vasodilatation*

Almost no alteration in vasomotor function was observed in isolated femoral arteries from EC-specific ETB deficient mice. Acetylcholine-stimulated vasodilatation, a commonly used measure of the 'endothelial dysfunction' seen in atherosclerotic and other diseased arteries (Freiman *et al.*, 1986; Bonthu *et al.*, 1997; Barton *et al.*, 1998; Ross, 1999; Monnink *et al.*, 2002) is perhaps the most relevant in the context of vascular injury. This process can be attributed to production of the vasodilator radical NO, as indicated by its near complete blockade by the NO synthase inhibitor, L-NAME, and was not altered by EC ETB knockout. As relaxations to the NO donor sodium nitroprusside were similarly unaffected, the absence of defects to this response cannot be explained by sensitisation of VSMCs to NO, as is reported in other situations of chemical or physical endothelial inhibition/damage (Moncada *et al.*, 1991). Acetylcholine-stimulated relaxation did appear to be impaired by ageing, consistent with previously reported defects in the NO pathway in aged mouse arteries (Blackwell *et al.*, 2004; Gendron *et al.*, 2006). This age-dependent dysfunction was not, however, exacerbated by EC ETB knockout. EC ETB, therefore, does not appear to modulate the bioactivity of NO or stimulated release of NO in the femoral artery, even in the presence of pre-existing endothelial dysfunction. Indeed, the only possible indication of altered endothelial function in these studies was an increased responsiveness of FF/tie2 vessels to the α_1-adrenoceptor agonist phenylephrine.

Given that inherent contractility, as measured by contraction to K^+, was not altered by genotype, this might indicate a reduction in the physiological antagonism of this constrictor response provided by basal or α_1-stimulated release of NO (Bullock *et al.*, 1986; Waldron *et al.*, 1999; Hadoke *et al.*, 2001). An alternative mechanism could be that EC ETB knockout has a more specific action on α_1-mediated vasoconstriction. Indeed, low concentrations of ET-1 (as might be produced by impaired ET-1 clearance in EC ETB deficient mice) are known to sensitise vascular rings to noradrenaline (Tabuchi *et al.*, 1989; Yang *et al.*, 1990).

These data describing the absence of effect of EC ETB deficiency on acetylcholine-induced vasodilatation contrast with those previously reported in aortic rings from both EC ETB knockout mice (Bagnall *et al.*, 2006) and transgenically-rescued ETB knockout mice (Quaschning *et al.*, 2005). Aortas from EC ETB-deficient mice were, therefore, examined in an attempt replicate these findings. No impairment to endothelium-dependent relaxation was seen with only the constrictor-phase of the response to acetylcholine being altered by EC ETB knockout. This enhanced contraction may, however, reflect an impaired ability of the endothelium to physiologically antagonise the constrictor component of the response to acetylcholine in FF/tie2 mouse aortas. This might be either due to loss of basal EC ETB-stimulated NO release or increased production of oxidative species by NAD(P)H oxidases (Duerrschmidt *et al.*, 2000; Wedgwood *et al.*, 2001; Dong *et al.*, 2005; Rodriguez-Vita *et al.*, 2005). This interpretation is supported by the identical constrictor response to acetylcholine between genotypes in vessels where NO production was blocked by pre-treatment with L-NAME. As such, alternative explanations such as altered VSMC muscarinic receptor function or enhanced cholinesterase activity appear less likely. It is not clear, however, why the impaired acetylcholine-mediated relaxation reported previously in these mice (Bagnall *et al.*, 2006) was not fully replicated in this investigation.

The remaining discrepancy of the complete absence of changes to endothelial function in the femoral artery, as compared to the modest alterations in the aorta has several possible explanations. This may indicate a different phenotype of endothelial

cells between vascular beds, either with regard to ETB expression, or pro-/anti-oxidant systems. Differences in mediators of endothelium-dependent vasodilatation between aortas and femoral arteries may also provide explanation as Crauwels *et al* (2000) report that the response to acetylcholine in the femoral artery is partly meditated by endothelium-derived hyperpolarisation. This is not supported by the data presented here, however, as in both arteries, L-NAME was seen to almost completely abolish this dilator response indicating complete-dependence on NO synthase activity.

5.4.2 *ET receptor distribution in the mouse femoral artery*

An additional aim of studies of vascular function was to clarify the distribution of ET receptor subtypes in the mouse femoral artery. Deficiency of ETB from EC had little effect on the constrictor response to ET-1 suggesting that, in the mouse femoral artery, expression and/or function of the ETB receptor in endothelial cells is weak. This may also reflect the rather crude nature of this measure – a composite of a powerful ETA/B-mediated constrictor response and an ETB-mediated dilator response, which in mouse vascular rings, is typically small, short-lived and inconsistent (Mizuguchi *et al.*, 1997; Zhou *et al.*, 2004). To address this issue, the response of pre-contracted vessels to a single dose of the ETB agonist, sarafotoxin S6c, was recorded. The responses elicited however were impossible to interpret - being extremely variable in size and direction and not altered in a consistent way by EC ETB deficiency (or endothelial denudation). In contrast, a clear constrictor response to 10^{-7}M sarafotoxin S6c was observed in mouse tracheal rings, which was sensitive to ETB blockade. The inability to observe this notoriously difficult to measure response, is not necessarily meaningful.

The distribution of contractile ET receptors proved easier to determine. ET-1-induced vasoconstriction was inhibited by a selective ETA antagonist in a manner consistent with the competitive nature of this agent (parallel rightward shift) but was not altered by a selective ETB antagonist. When applied to L-NAME treated, non-contracted femoral arteries, stimulation with an ETB agonist did elicit a small but obvious constrictor response. These data are consistent with the idea ETA is the

dominant vasoconstrictor receptor for ETs in conduit arteries (Davenport *et al.*, 1995a; Davenport *et al.*, 1995b), and confirms the expression of ETA and to a lesser extent ETB by femoral artery smooth muscle cells. It may also be that a population of ETB receptors is normally quiescent, and in this case, become active only in the presence of ETA blockade. Such a cross-talk phenomenon has been previously reported, for example, in rat mesenteric arteries (Mickley *et al.*, 1997).

Neither the response to ET-1 in the presence of ETA blockade nor that to an ETB agonist in non-contracted femoral artery rings was altered by EC ETB deficiency. This suggests that ETB-deficiency specifically in EC does not result in alterations to ETA and ETB receptor function in other cells. These observations are consistent with previous work demonstrating that, in all tissues, binding of an ETA-specific and, in non-EC rich tissues, of an ETB-specific radioligand to whole body autoradiographs is not altered in EC ETB-deficiency (Kelland *et al*., Unpublished observations.). This is somewhat surprising given that ETA-mediated vasoconstriction is substantially impaired (~50% of control) in aortic rings from transgenically-rescued, non-cell-specific ETB knockout mice, independent of blood pressure (Quaschning *et al.*, 2005). Reduced ETA receptor expression is also reported in the kidneys of ETB knockout rats (Taylor *et al.*, 2003) and the lung and brain of neonatal $ETB^{-/-}$ mice (Davenport & Kuc, 2004; Kuc *et al.*, 2006). In each model this is suggested to be a consequence of chronic exposure to the elevated plasma ET-1 concentrations associated with ETB deficiency. EC-specific ETB knockout mice (Bagnall *et al.*, 2006) and EC-specific ET-1 over-expressing mice (Amiri *et al.*, 2004), however, have similarly elevated circulating ET-1 levels. In these models, no down-regulation of vascular ETA receptors is apparent. Smooth muscle cell ETA receptor expression may, therefore, be directly regulated by ETB by an intracellular mechanism rather than indirectly via exposure to excess ligand. This is consistent with work by Davenport & Kuc (2004) who report, in ETB-deficient rats, that ETA down-regulation is more pronounced in tissues which would normally strongly co-express both receptor subtypes. An alternative explanation lies in the distribution of ET-1 clearance receptors. EC ETB appears essential to the removal of ET-1 from plasma (Fukuroda *et al.*, 1994a; Johnstrom *et al.*, 2005; Bagnall *et al.*,

2006; Kelland et al., Unpublished observations). Deeper within tissues, such as in the arterial media, however, ETB expressed by other cells types, such as VSMC may be more important in determining local concentration. As such, whilst plasma levels are similar between EC-specific and non-specific models of ETB deficiency, local tissue concentrations in the vascular wall may be much greater in non-specific ETB knockout animals and elicit compensatory down-regulation of ETA.

5.4.3 *EC ETB deficiency does not alter the neointimal lesion size*

The weak effects of EC ETB deficiency on femoral artery vascular function may go some way to explain data from vascular injury studies. No effect of EC-specific ETB receptor deletion on any histology- or OPT-derived measure of neointimal lesion size following wire-induced injury, the primary endpoints of these studies, was observed. This contrasts with the data in chapter 4, which demonstrates that non-cell-specific ETB blockade increases lesion size following femoral artery wire-injury. Any effect on the rate of lesion progression, perhaps a more sensitive measure, is also unlikely: lesion size was equally unaffected by EC ETB deficiency at 14 days post-surgery, a known time-point of intermediate lesion size (Sata *et al.*, 2000), and no difference in proliferating cell count was noted. These data support the obvious conclusions that ETB in mouse femoral artery endothelial cells does not exert a moderating effect on neointimal thickening and, therefore, that the effects of ETB blockade described in chapter 4 are mediated by a receptor expressed by a non-endothelial cell type. They might also provide further evidence against a role for impaired ET-1 clearance and resulting ET-1 excess in the effects of pharmacological (chapter 4) and genetic (Murakoshi *et al.*, 2002) inhibition of ETB on neointimal lesion formation. Plasma ET-1 concentrations are similarly elevated between EC-specific ETB deficient mice and rats treated with 30mg/kg/day A192621 or deficient in ETB (Gariepy *et al.*, 2000; Opgenorth *et al.*, 2000; Bagnall *et al.*, 2006). Only non-specific ETB blockade/deletion, however, is associated with increased neointimal thickening following vascular injury. As discussed in the context of ETA receptor expression above, one likely caveat to this interpretation is the possibility that these two manipulations might have a differential effect on local tissue concentrations of ET-1. Indeed, neointimal lesions are typically reported to possess high densities of both

ETB expressing and ET-1 secreting cells implying that intra- lesion ET-1 levels may be regulated by non-EC ETB pathways (Dashwood *et al.*, 1999; Shirai *et al.*, 2006).

As in the wire injury model, neointimal lesions arising from ligation of the femoral artery were unaffected by EC ETB deficiency at 28 days. In this case, these data are in agreement with a similar absence of effect of pharmacological ETB blockade on neointima formation in this model (chapter 4). This indicates that the inability of ETB blockade to increase neointimal lesion size following femoral artery ligation-injury is not the result of competition between EC-mediated, lesion-moderating actions (such as NO release), and non-EC-mediated lesion-enhancing actions (such as mitogenesis/inflammation).

To address any effect of EC ETB deficiency on the rate of lesion development in this model, ligation-injured vessels were also analysed at 14 days. This was predicted to represent a time point of active lesion growth, as it does both in femoral artery wire-injury (Sata *et al.*, 2000) and in carotid artery ligation-injury models (Godin *et al.*, 2000). No differences in lesion size or composition, however, were apparent between 14 and 28 days post-ligation suggesting that complete femoral artery ligation-induced lesions stabilise in size within 14 days. This may reflect the smaller size of lesions induced by this method (similar in cross-sectional area to wire-induced lesions at 14 days) or the severity of blood stasis/flow disturbance caused by complete ligation of this major artery.

Injury-induced changes in endothelial cell phenotype might explain why EC ETB-deficiency is unable to alter the response to vascular injury in these models. Certainly, the data presented in chapter 3 indicate that ligation-injury is associated with impairment of acetylcholine-stimulated vasodilatation. In the wire-injury model, whilst investigations of vascular function have been prevented by the unavoidable severe medial damage that is implicit to this injury, it seems likely that a rapidly re-grown endothelium, possibly of progenitor cell origin, might have at least some altered properties. In support of this, whilst Miller *et al* (2003) showed the return of endothelium-dependent vasodilatation to Ca^{2+} mobilisation by cyclopiazonic acid

within 10 days of *in vivo* endothelial denudation in the mouse the carotid artery, complete muscarinic receptor stimulated vasodilator responses were slower to return. Any relevance of this issue, however, is restricted to the matter of mechanism as various models of endothelial dysfunction are associated with increased neointimal lesion size in femoral artery wire-injury and carotid artery ligation models (Wang *et al.*, 2005a; Zhang *et al.*, 2006). Further, any manifestation of neointimal hyperplasia in man, such as post-interventional restenosis or vein graft disease, is likely to be associated with endothelial damage or dysfunction.

It must also be considered that in other experimental models or situations, EC ETB might play a greater role. Foremost, it has not been possible to prove the presence of ETB in the femoral artery endothelium or observe any effect of ETB deficiency on endothelial cell function in this vessel. In some cases, this may simply reflect experimental limitations but may also suggest that the role or expression of EC ETB in femoral arteries is not representative of all murine vessels. This would certainly offer an explanation for the differing effects of pharmacological ETB blockade on neointimal hyperplasia following carotid (increased; Murakoshi *et al.,* 2002) and femoral artery ligation (no effect; chapter 4). Species differences might also warrant consideration as, whilst EC ETB receptors undoubtedly exist in the mouse, in this species administration of an ET-1 bolus elicits only vasoconstriction (Giller *et al.*, 1997). This may indicate that, in the mouse, either EC ETB function or distribution is not representative of that in larger animals and as such, that in these species, EC ETB might exert a more obvious effect of neointimal proliferation.

5.4.4 *EC ETB regulates femoral artery composition following wire-injury*

Despite the apparent inability of deletion of ETB from ECs to alter lesion size, 14 days following injury, increased intimal α-smooth muscle actin immunoreactivity was observed. This was not accompanied by increases in the number of proliferating cells suggesting either changes in cell size or increased survival rather than proliferation. Alternatively, it may be that a transient increase in cell proliferation rate has normalised by 14 days. Whatever the mechanism, the result is a lesion with a more mature appearance, more similar to those in FF/-- mice at 28 days than 14 days

following injury. Thus, although lesion size was not altered, the rate of maturation may be reduced by influences of EC ETB. This may be because vascular NO, which suppresses smooth muscle migration and proliferation (Garg & Hassid, 1989), is reduced by EC ETB-deficiency. These compositional changes were apparent at 14 days, however, suggesting that they are not mediated by an effect of deletion from the endothelial cells lining the femoral artery lumen as EC function probably only returns by day 10 (Liao *et al.*, 2007). As such, these effects may reflect a response to a systemic phenotype such as elevated circulating ET-1 concentrations, which are inherent to EC ETB knockout mice (Bagnall *et al.*, 2006). ET-1 is known to promote both smooth muscle proliferation (Komuro *et al.*, 1988) and, when acting on the ETA receptor, to protect smooth muscle cells from a variety of pro-apoptotic influences (Diep *et al.*, 2000; Shichiri *et al.*, 2000; Wu-Wong *et al.*, 2000). Excess of ET-1 may also stimulate hypertrophy of smooth muscle cells, and this process is stimulated by lower concentrations of ET-1 than is necessary to promote mitogenesis (Dao *et al.*, 2006). This may be of particular relevance as levels of ET-1 in plasma, even in (EC) ETB deficient mice, are extremely low (Bagnall *et al.*, 2006). This leaves the question of why an apparent increase in smooth muscle cells manifests as altered composition rather than lesion size. It may be that smooth muscle cells can migrate into a relatively acellular framework (fibrin, platelets, extracellular matrix) at this time point without requiring concurrent lesion expansion (Schwartz *et al.*, 1992a). EC ETB deficiency may also negatively regulate a non-smooth muscle lesion component that has not been analysed such as elastin or ground substance. Alternatively, any differences seen may reflect an effect on the level of αSMA expression in otherwise normal smooth muscle cells rather than an increase in their number or size.

Even greater increases in αSMA immunoreactivity were noted in the media of wire-injury femoral arteries from EC ETB-deficient mice. This increase was observed at both 14 and 28 days post-injury and may explain changes observed in neointimal composition as they result in a larger pool of smooth muscle cells that can migrate into the developing neointima. In this model, the severe stretch associated with wire insertion induces rapid apoptosis of medial smooth muscle cells, resulting in a

dramatic loss of these cells within 24 hours (Sata *et al.*, 2000). It therefore seems likely that the mechanism for this effect of EC ETB deficiency is the inhibition of stretch-induced apoptosis. These changes were not associated with increased BrdU incorporation further implying an effect on cell survival rather than growth. The strength of this effect at 14 days, once again suggests that a systemic effect of EC ETB knockout, such as impaired plasma ET-1 clearance, rather than a local effect of femoral artery luminal ECs, is responsible. The same mechanisms which potentially contribute to increased neointimal smooth muscle-like cell content are all possible: an anti-apoptotic action of ETA activation, hypertrophy, proliferation prior to day 14 and changes in αSMA expression levels. Both intimal and medial compositional changes in EC ETB knockout mouse wire-induced lesions may also be explicable by enhancement to the recruitment or differentiation of circulating progenitor cells to the injured vascular wall. At first glance this is consistent with the absence of such changes in the lesions of ligation-injured vessels, which are anticipated to develop without contribution of circulating progenitors (Tanaka *et al.*, 2003). This discrepancy between models, however, equally implicates any altered response to the medial damage that occurs in wire but not ligation-injury including an anti-apoptotic action. Regardless, it is interesting that no such changes in medial composition at 28 days post-wire-injury were present in ETB antagonist treated mice (chapter 4). It is not clear whether this indicates that the mechanism is mediated by non-EC ETB receptors and is, therefore, prevented by non-specific ETB blockade, or that the effect is masked by whatever process results in increased lesion size following this treatment.

These alterations to medial composition may also offer explanation for the small, but significant, increase in total luminal volume observed at 14 days following wire-injury in EC ETB knockout mice. At this time point, because neointimal formation is not extensive, luminal volume is determined in large part by medial structure. As wire-injured arteries from this point are extremely fragile, even following perfusion fixation, an increase smooth muscle-like cells in the media may protect 14 day arteries from FF/tie2 animals from compression of the lumen during tissue preparation and handling. The alternative, that this finding reflects arterial

dilation/outward remodelling, is unlikely. No changes in the area inside the internal elastic lamina (neointima + lumen) were present in histologically-evaluated sections. These measurements were made on the largest neointimal lesions from each artery, and therefore, describe the regions most resistant to handling artifacts.

5.4.5 *Non-EC ETB receptors moderate neointimal proliferation?*

If the increases in neointimal lesion size observed following pharmacological ETB blockade cannot be explained by actions of this receptor expressed by ECs, then ETB expressed by other cell types must be responsible. An effect on vascular smooth muscle cells is certainly a reasonable proposition given that these cells are not only the most numerous in the developing and mature neointima but are thought to strongly express ETB (Azuma *et al.*, 1994; Azuma *et al.*, 1995a; Dashwood *et al.*, 1999; Shirai *et al.*, 2006). Indeed, myography studies in this chapter have shown that even in the uninjured femoral artery, smooth muscle cells express functional ETB receptors. ETB receptors, in these cells, may promote apoptosis, including that induced by stretch injury. This could explain why ETB blockade but not EC ETB-deficiency increases lesion size following wire-injury. Certainly, continual apoptosis of neointimal cells has been noted in this model (Reis *et al.*, 2000; Sata *et al.*, 2003) and modification of apoptosis appears to regulate lesion growth (Blanc-Brude *et al.*, 2002; Sata *et al.*, 2003; Tanja Rauma-Pinola, 2006). Whilst convenient, this explanation is somewhat contradictory to the suggestion that an ETA-mediated, anti-apoptotic effect regulates lesion composition in EC ETB knockout mice. Many other explanations can be speculated such as a negative regulation of macrophage/neutrophil function by ETB, an effect on recruitment of smooth muscle progenitor cells to the neointima or induction of NO production in smooth muscle cells. Further study is, therefore, required to determine the mechanism by which non-EC ETB moderates the response to vascular injury.

5.4.6 *Experimental limitations*

It must be considered that the inability of EC ETB deficiency to alter neointima formation following femoral artery wire-injury could be a consequence of limitations

of the experimental approach, rather than truly reflecting physiological processes. In these mice, specific deletion of the ETB receptor gene is dependent on robust expression of the Tie2 promoter. Following the initial removal of endothelium in this model, it may be that the ECs that re-grow do not express Tie2 as might be expected. This seems unlikely considering the role of the angiopoietin-1/Tie2 system in angiogenesis and EC maturation (Suri *et al.*, 1996; Wong *et al.*, 1997), but the possibility exists that the absence of Tie2 expression in the neo-endothelium allows these cells to retain ETB expression. Similarly, the specificity of deletion to EC is dependent on the expression of Tie2. Reporter animals suggest that the vast majority of Tie2 expression occurs in EC; however, both expression of Tie2-Cre mRNA and Tie2-Cre mediated recombination have been suggested to occur in cells of hematopoietic lineage (Constien *et al.*, 2001). In the context of wire-injury-induced neointimal lesions, this may result in ETB deletion from macrophages and neutrophils, both critical to lesion formation. Zhao *et al* (2006), however, report that Tie2-Cre mediated recombination is absent in bone marrow lymphocytes and in total white blood cells. Unlike the work of Constien *et al* (2001), this study utilised the same transgenic Tie2-Cre line used to create EC ETB-deficient mice. Further, recent work in this laboratory has demonstrated that macrophages isolated from EC ETB-deficient mouse bone marrow, retain ETB expression (Azfer *et al.,* Unpublished observations). Smooth muscle like-cells derived from circulating progenitors may also be affected, although the early work that suggested these are of hematopoietic origin (Sata *et al.*, 2002) has since been called into doubt (Sahara *et al.*, 2005). The possibility cannot be completely excluded, however, that a beneficial effect of EC ETB deficiency is masked by unintended ETB deficiency in other cell types important to lesion development.

Finally, the comparison of the effects of pharmacological ETB blockade (chapter 4) and EC-specific ETB deficiency on neointimal lesion formation may be complicated by the effects of genetic background. EC ETB knockout mice used in this chapter are of a mixed strain comprising 129/Ola and BKW. Studies of the effects of ET antagonists on lesion formation (chapter 4), and of femoral artery injury on vascular function (chapter 3), however, were performed in mice of the strain C57Bl/6J (for

unavoidable practical reasons). It is possible, therefore, that the effects of ETB blockade observed in chapter 4 are indeed mediated by ETB receptors on EC, and that the negative results presented here are a consequence of differences in the role of ETB in the response to acute vascular injury between these two strains.

5.4.7 *Conclusions*

The data presented in this chapter indicate that, whilst femoral artery endothelial function is not overtly regulated by endothelial ETB activity, regional differences exist in the response of EC to ETB deletion. It is perhaps not surprising, therefore, that no differences in neointimal proliferation were associated with EC ETB deficiency in two models of vascular injury to this artery, contrary to the original hypotheses of this work. Whilst effects on lesion composition are interesting, they are perhaps of limited consequence considering that neointimal lesions are inherently stable. The most interesting conclusions come with comparison to the striking effects of pharmacological ETB blockade on wire-injury-induced neointimal lesions. In this context, regardless of the reasons for the ineffectiveness of EC ETB deficiency (lack of role in vascular function, wire-induced endothelial denudation), in this model, in this artery, in this species, a non-EC ETB receptor appears to offer protection against neointimal thickening following acute injury. Future work should address the cell types and mechanisms mediating this effect of non-specific ETB blockade and may predict how applicable these data are to other processes of vascular remodelling.

Chapter 6

Conclusions and future directions

6.1 GENERAL CONCLUSIONS

Acute injury to the vascular wall, such as that inflicted by percutaneous coronary intervention, elicits formation of neointimal lesions by activation of inflammatory and proliferative cascades. Although often an appropriate healing response, excessive neointima formation can compromise vessel patency and thus represents a major limiting factor to the success of such procedures. It is well established that the endothelin (ET) system contributes to this process. In animal models, neointima formation is enhanced by administration of exogenous ET-1 (Trachtenberg *et al.*, 1993; Barolet *et al.*, 2001) and diminished by blockade of the ETA receptor, consistent with the pro-mitogenic, pro-inflammatory activity of the ET-1/ETA interaction (Ferrer *et al.*, 1995; Takiguchi & Sogabe, 1996; Burke *et al.*, 1997; McKenna *et al.*, 1998; Dashwood *et al.*, 1999; Murakoshi *et al.*, 2002; Tepe *et al.*, 2002; Wan *et al.*, 2004). What is less clear is whether concurrent activation of the ETB receptor also contributes to lesion development. Certainly, ETB mediates many detrimental actions common to ETA such as induction of smooth muscle proliferation (Eguchi *et al.*, 1994). ETB may, however, exert other effects that might limit lesion formation such as stimulating production of nitric oxide (NO; Hirata *et al.*, 1993; Tsukahara *et al.*, 1994; Mizuguchi *et al.*, 1997), a potent inhibitor of neointima formation (Moroi *et al.*, 1998; Zhang *et al.*, 2006; Cooney *et al.*, 2007) and clearance of circulating ET-1 by endothelial cells (ECs; Fukuroda *et al.*, 1994a; Bagnall *et al.*, 2006). Further, ETB receptors may promote apoptosis of vascular smooth muscle cells (VSMC; Cattaruzza *et al.*, 2000; Lauth *et al.*, 2000) and exert a tonic hypotensive effect by the regulation of renal sodium handling (Ge *et al.*, 2006). Although much literature indirectly suggests that any role for ETB in neointima formation is minor, it has recently been reported that carotid artery ligation-induced neointima formation is accelerated in ETB-deficient mice (Murakoshi *et al.*, 2002). The work presented in this thesis was, therefore, undertaken to clarify the influence of individual ET receptor subtypes in neointimal lesion formation following acute injury to the arterial wall, in particular what role is played by ETB in EC in this setting?

6.1.1 *Induction of vascular injury*

An obvious prerequisite for any such investigation is the ability to model and quantify the response to vascular injury. Two models of acute injury to the mouse femoral artery have been previously utilised in this laboratory for similar studies. The first, a widely used model of intra-luminal wire injury that mimics the nature of injury associated with PCIs in man and is known to provide a strong stimulus for neointima formation (Sata *et al.*, 2000). The second, a variation on the wire-injury sham operation in which ligation of the popliteal artery, just distal to its junction with the femoral artery is sufficient to induce neointima formation. The lesions resulting from this latter injury, however, are small, inconsistent and extremely focal (MacDonald *et al*, Unpublished data). In an effort to develop a more robust ligation-induced model of neointima formation in the femoral artery, alternative ligations to the femoropopliteal bifurcation were investigated. It was found that ligation of the femoral artery immediately distal to this junction ('partial' ligation) also provoked formation of only small, focal neointimal lesions. In contrast, ligation of the femoral artery proximal to its bifurcation ('complete' ligation) elicited development of larger neointimal lesions over greater lengths of vessel - more reminiscent of those reported to occur in response to ligation of the carotid artery (Kumar & Lindner, 1997). Thus, both the femoral artery wire-injury and complete ligation-injury models were adopted for subsequent studies. These models were complementary in several ways and by performing both in the same animals the amount of information gleaned from each was increased. Perhaps the most critical difference between the two injuries is the denudation and re-growth of endothelial cells implicit in the wire- but not ligation-injury model. Any interpretation of data with regard to a hypothesised role of ETB in EC on neointima formation was, therefore, considered to be more straightforward following ligation-injury. These models also importantly differ in the origin of cells contributing to neointima formation. Wire-injury results in recruitment of bone marrow-derived progenitor cells to the neointima, whereas this does not appear to occur in ligation-injury models in which neointimal smooth muscle cells are predicted to derive from the media (Tanaka *et al.*, 2003).

In both models, studies were undertaken to investigate vascular function in injured arteries with two major aims. First, to confirm the integrity of endothelium-dependent responses after injury, and in the case of wire-injury, ensure recovery of endothelial function correlates with physical re-growth. Second, to determine if injured vessels exhibit altered functional response to ET-1 that may modify their role in the response to vascular injury. In wire-injured vessels, any determination of these parameters was prevented by the complete inability of injured femoral artery rings to develop any contractile force – probably reflecting the severity of injury to the media. Functional studies of uninjured vessels conducted in parallel, however, provided basic proof for the expression of ET receptors in femoral artery smooth muscle cells, and that the constrictor response to contractile agonists including ET-1 was basally suppressed by the endothelium.

Complete ligation-injured vessels exhibited comparatively mild functional changes. Although vessels dilated in response to acetylcholine, a moderate dysfunction of this endothelium-dependent response was present. An altered smooth muscle phenotype was also apparent as absolute responsiveness to a number of vasoconstrictor substances was impaired, although no more so for ET-1 than for other agents. Studies of partial ligation-injured vessels, in which no appreciable neointima formation is observed, indicated similar functional changes. In these too, endothelium-dependent vasodilatation and absolute contractility were moderately impaired. As such, these changes appear to occur independently of neointima formation and are perhaps a response to altered blood flow patterns and shear stresses around the site of manipulation, which are well established to regulate vascular structure and function (Caro *et al.*, 1971; Cunningham & Gotlieb, 2005).

6.1.2 *Measurement of neointimal lesions*

Previous experience with the wire-injury model also highlighted the limitations of traditional histological means for the quantification of neointimal lesion burden. Microtomy, histological staining and lesion analysis of long lengths of injured femoral arteries is extremely laborious and time-consuming. Moreover, this process

prevents any accurate volumetric measurement of lesion burden because even careful serial sectioning does not truly retain z-axis information and position in 3D space.

Optical projection tomography (OPT) was explored as a potential means to rapidly image isolated arteries in 3D for qualitative and quantitative assessment of morphology along the entire vessel. This proved extremely successful, with OPT scans bearing striking resemblance to subsequent histological staining of the same regions. Moreover, quantitative measurements of 2D lesion size compared well between the two techniques and, as such, it was possible to make volumetric measurements and cross-sectional profiles of luminal and lesion size. OPT scans, therefore, provided a valuable complement to histological analysis of neointimal lesion formation, providing additional power to detect the effects of interventions, and describing the distribution of lesion formation – data which would otherwise have been lost. Further, this technique may be considered particularly important because few alternative methods for the 3D evaluation of small arteries have been described, and those that have, carry severe practical limitations (McAteer *et al.*, 2004).

6.1.3 *Role of the ETA receptor*

Whilst the role of the ETA receptor in vascular remodelling following acute arterial injury has been previously demonstrated, it was necessary to confirm that this receptor plays a similar role in the femoral artery wire- and ligation-injury models. This was achieved by administration of a highly selective ETA receptor antagonist (atrasentan, 2000-fold ETA/ETB selective) to animals subject to femoral artery wire and ligation injury. The results of these experiments, as expected, indicated that ETA blockade reduced several measures of wire-injury induced lesion size and volume at a time point of stable lesion size and dramatically preserved the luminal cross-section. In comparison, histological measures indicated no effect of ETA blockade on ligation-induced lesion formation. Analysis by OPT, however, demonstrated that ETA blockade reduced lesion size in the axial dimension, and, therefore, lesion volume; confirming both a role for ETA in this model and demonstrating one of the potential advantages of OPT. Whilst the mechanism of this action was not explicitly

investigated, no differences in lesion composition due to ETA blockade were apparent in either model. It must be noted, however, that lesion macrophage content and cell proliferation, two likely candidates for modulation by ETA, are poorly represented by the time-point investigated. Thus, activation of the ETA receptor by endogenous ETs is important in the development of neointimal hyperplasia following both femoral artery wire-injury and ligation-injury. This further supports the rationale for use of ETA antagonists in the prevention of similar remodelling processes in man, and provides validation of the experimental design for studies investigating the effects of the ETB blockade.

6.1.4 *Role of the ETB receptor*

The effect of ETB blockade on lesion formation was of greater uncertainty and a primary topic of this work. ETB blockade was achieved in a similar manner to ETA blockade – by administration of a selective antagonist (A192621, 1300-fold ETB/ETA selective) to mice subject to femoral artery wire- and ligation-injury procedures; either alone or in combination with ETA blockade. Neointimal lesions induced by wire-injury were substantially increased, both in size and in volume, by selective ETB antagonist treatment. An increase in neointimal lesion size with ETB antagonist treatment was apparent even in the presence of concurrent ETA blockade. Lesions in mice treated with the combination of ETA and ETB antagonists were of similar size to those from vehicle-treated animals and were, therefore, increased in size relative to those from mice subject to ETA blockade alone. These data serve to confirm the work of Murakoshi *et al* (2002) and demonstrate that the ETB receptor acts to moderate neointima formation following vascular injury. As such, the ETB receptor appears to act to limit the detrimental effects of ETA activation by endogenous ETs in this process. The ability of ETB blockade to increase lesion size, even in the presence of ETA blockade suggests that the action of ETB antagonism in this setting is not simply a response to elevations in blood pressure or in plasma [ET-1], two well characterised systemic effects of this treatment. Analysis of lesion composition again provided little conclusive evidence with regard to mechanism of action, although increases in cell proliferation, and perhaps macrophage content were apparent in lesions from ETB antagonist treated mice. Such a pro-mitogenic, pro-

inflammatory lesion phenotype would be consistent with inhibition of the EC ETB / NO pathway, as NO release inhibits inflammatory (Bath *et al.*, 1991) and mitogenic processes (Garg & Hassid, 1989). Analysis of lesion composition at earlier time points of active lesion growth would be necessary to confirm these changes.

In contrast to the positive results with ETB blockade on wire injury-induced lesion formation, little effect on lesion size or volume was observed with this treatment in the ligation-injury model. Similarly, when co-administered with an ETA antagonist, lesion volume was reduced in this model to a similar extent as for ETA blockade alone. Therefore, in the femoral artery ligation-injury model, ETB receptors have little role in lesion development and do not modulate the response to ETA blockade. This apparent difference in the actions of ETB between wire and ligation induced-injures is surprising for several reasons. First, Murakoshi *et al* (2002) describe reduced lesion size in the carotid artery ligation model with ETB deletion or blockade. This may reflect differences in ETB expression or VSMC phenotype between carotid or femoral arteries, or perhaps the phase of lesion growth analysed - is growth accelerated or increased in maximum stable size? Second, if ETB blockade increases lesion size by an action on EC, one might predict a greater effect in the ligation model in which EC are not denuded, than the wire-injury model. This might indicate the inhibitory effect of ETB on lesion formation is not mediated by EC but many other differences between models may also account for this discrepancy. For example, ETB may inhibit recruitment of bone marrow progenitors to wire-induced neointimal lesions or stretch-induced VSMC apoptosis, both processes that would not be expected to occur in the ligation model (Tanaka *et al.*, 2003). Such variability between models at least may explain why many, but not all, publications report that both ETA selective and non-selective ETA/B antagonists are effective at reducing neointima formation.

6.1.5 *Role of the ETB receptor in EC*

The most compelling explanations for the observed moderating action of ETB on wire-induced lesion formation depend on actions of this receptor in EC such as stimulation of NO release (Hirata *et al.*, 1993; Tsukahara *et al.*, 1994; Mizuguchi *et*

al., 1997), EC mitogenesis (Goligorsky *et al.*, 1999; Salani *et al.*, 2000; Kuhlmann *et al.*, 2005) and ET-1 clearance (Dupuis *et al.*, 2000; Bagnall *et al.*, 2006). Pharmacological tools cannot distinguish between ETB receptors expressed by different cell types, necessitating the use of cell-specific ETB deletion to further unravel the role of ETB. To understand how EC ETB might alter femoral artery function (and therefore neointima formation), the vasomotor responses of isolated femoral artery rings from EC ETB knockout animals were determined. Few disturbances to vascular function were apparent, importantly including endothelium-dependent vasodilatation and vasoconstriction to ET-1. This contrasts with previous studies of the aorta of these mice, which were partially reproduced here, and indicate impaired vasodilatation to acetylcholine (Bagnall *et al.*, 2006). Direct stimulation of ETB produced inconsistent responses, most reliably eliciting vasoconstriction. Together these data suggest that the function of EC ETB varies between vascular beds and is minor in the mouse femoral artery. It must be considered, however, that previous experience in this laboratory and inconsistency in the literature (Mizuguchi *et al.*, 1997; Zhou *et al.*, 2004) suggest that responses to ETB activation are difficult to reproduce in mouse vascular rings and as such may represent experimental limitations rather than a true physiological role.

Femoral artery wire-injury was performed to determine whether deletion of ETB from EC alone could recapitulate the effects of pharmacological ETB blockade. Similarly, ligation-injuries were also studied to reveal whether deficiency of ETB specifically from EC alters lesion formation; for example, if a detrimental effect of ETB in VSMC physiologically antagonises a beneficial effect of ETB in EC. In neither model, however, was EC ETB deficiency found to be associated with any difference in measures of lesion size or volume. More subtle alterations to wire injury-induced lesion composition were noted, however, and possibly indicate that EC ETB, by some means, acts to delay lesion maturation and enhance apoptosis of medial VSMC. Regardless, these data suggest that in both models, any role for EC ETB in the regulation of neointima formation is small, which is perhaps unsurprising given the apparently minimal role of EC ETB in mouse femoral artery vascular function. Further, this contrasts with the strong effect of pharmacological ETB

blockade on wire-induced neointima formation (above). This suggest that ETB receptors expressed by other cell types offer protection against neointimal thickening in this model. Several possible alternative mechanisms for this response exist such as ETB-mediated inhibition of VSMC apoptosis (Cattaruzza *et al.*, 2000; Lauth *et al.*, 2000), or stimulation of eNOS activity in neointimal VSMC (Wang *et al.*, 2005a). Whilst these examples are purely speculative, this question of mechanism provides an intriguing avenue for further investigation.

6.1.6 *Relevance of findings*

Although the effects of selective ETB blockade on remodelling following vascular injury are mechanistically interesting, any clinical relevance is limited because no rationale exists for the use of the selective ETB receptor antagonists in cardiovascular disease patients. Selective ETA antagonists (sitaxsentan, ambrisentan) and non-selective ETA/B antagonists (bosentan), however, are both approved for use in pulmonary arterial hypertension, and are in clinical investigation for a variety of other conditions (e.g. systemic hypertension, chronic kidney disease; Battistini *et al.*, 2006). Neither antagonist strategy has been proved ultimately superior, despite much argument over the theoretical benefits of both treatment modalities (Bagnall & Webb, 2001; Battistini *et al.*, 2006). The data presented here demonstrate that concurrent ETB blockade can prevent the beneficial effects of ETA blockade on wire-induced neointimal lesion formation. It is, therefore, tempting to suggest that selective ETA receptor antagonists may be superior to non-selective ETA/B agents in the primary prevention of diseases which feature similar remodelling processes such as post-interventional restenosis or vein graft disease.

Beyond the primary prevention of vascular remodelling, a beneficial effect of ETA blockade on neointimal proliferation might indicate that selective ETA antagonists but not non-selective ETA/B agents have the potential to provide secondary benefits on other vascular proliferative/inflammatory processes, such as atherosclerosis. Whilst ET antagonists are never likely to be considered for primary prevention of atherogenesis, due to their profile of adverse effects, such an action may provide a secondary benefit where ET blockade is indicated for other reasons: such as the

treatment of resistant systemic hypertension (Battistini *et al.*, 2006). Certainly, selective ET antagonists are reported to be effective at reducing lesion size in murine models of atherosclerosis (Barton *et al.*, 1998; Babaei *et al.*, 2000). Whether the effects of ETB blockade on femoral artery wire-injury are relevant to this process is more speculative and undoubtedly dependent on the mechanism through which this treatment increases neointimal lesion formation. Blockade of the ETB-induced NO release pathway, for example, might be considered to exacerbate both neointimal hyperplasia and atherosclerosis because NO moderates the pathogenesis of both conditions (Moroi *et al.*, 1998; Kuhlencordt *et al.*, 2001; Zhang *et al.*, 2006). Conversely, inhibition of ETB-induced VSMC apoptosis might promote an unstable atherosclerotic plaque phenotype and, therefore, be detrimental. In this regard it is noteworthy that following wire-injury, combined ETA+ETB, but not selective ETA, blockade is associated with reduced intimal collagen and, perhaps, increased intimal macrophage content. In the setting of atherosclerosis both compositional changes might be considered to promote an unstable lesion phenotype. This question will ultimately be answered only with future experimentation (see below).

6.2 LIMITATIONS AND FUTURE EXPERIMENTS

The work described in this thesis has clarified the roles of ETA and ETB receptors, and ETB receptors expressed by EC in the response to acute vascular injury. Although it is apparent from these experiments that ETB receptors can exert a moderating effect on processes of vascular remodelling, the mechanism(s) by which they do so remain(s) unclear, providing an obvious avenue for future investigation. Further studies are also warranted both to provide additional experimental validation and to expand the scope of these findings.

6.2.1 *Further validation of EC ETB experiments*

Genetic background is an important determinant of gene expression patterns and cardiovascular phenotype. As such, all experiments in non genetically-modified mice were conducted in a single inbred strain (C57Bl/6J) known to be sensitive to wire injury-induced lesion formation (Roque *et al.*, 2000; Sata *et al.*, 2000). In studies of

EC ETB knockout mice, to control for variability in non-ETB gene loci, FF/tie2 (EC ETB-deficient) animals were directly compared with FF/-- littermates, in accordance with other models produced using the Cre-loxP approach (Liao *et al.*, 2001; Ge *et al.*, 2006). The background on which these animals were produced, however, differs from that used in other experiments in this thesis, complicating direct comparisons between these data sets. Specifically, it is plausible that while ETB blockade increases wire injury-induced lesion size in the C57Bl/6 strain, it does not do so in the BKW/Ola/129 strain in which EC ETB knockout was created. Therefore, that negative results in EC ETB-deficient animals could reflect a strain difference in ETB function or expression. Certainly, wire-injury elicited lesions at 28 days that were substantially smaller in vehicle treated C57Bl6/J mice (42900 ± 3600 μm^2; Chapter 4) than in either FF/-- (73660 ± 14650 μm^2) or FF/tie2 mice (70490 ± 13190 μm^2; Chapter 5). Intima/media ratios, however, were similar between C57Bl/6J (3.1 ± 0.3), FF/-- (3.22 ± 0.73) and FF/tie2 (3.09 ± 0.40) mice suggesting that such differences are a reflection of the smaller size of the C57Bl/6J strain. To address this question, further experiments involving the administration of A192621 to FF/-- mice are required. Repeated back-cross (>10 generations) of FF/-- and FF/tie2 animals on to the C57Bl/6J background would equally allow this question to be answered. Although this process is extremely time consuming, it would allow future experiments with FF/tie2 mice to utilize non-floxed 'wild-type' animals as an additional control and therefore enable any effect of the insertion of loxP sites in the ETB gene locus on outcome to be determined.

An additional remaining question over the use of EC ETB knockout mice for vascular injury studies concerns the expression pattern of Tie2, on which specific deletion of ETB is dependent, by cells of the neointima. It is plausible that the EC that re-grow following wire-injury and denudation do not express Tie2. It is also possible that Tie2 may be expressed at some point during the lineage of both leucocytes and neointimal VSMC-like cells derived from circulating progenitors, as Constien *et al* (2001) suggest that this gene is expressed by cells of hematopoietic lineage. Both of these possibilities could explain the negative results from these studies, and could be confirmed or excluded by performing vascular injury

procedures in a Tie2 promoter reporter animal: for example, that produced by the cross of flox-STOP-flox, lacZ or GFP reporter animals and FF/tie2 mice.

6.2.2 *Mechanism of ETB action*

The completion of further validating experiments aside, perhaps the most interesting avenue for further investigation is that of the mechanism by which non-EC ETB receptors might act to moderate neointimal proliferation. Although basic lesion composition in ETB antagonist-treated mice was examined here, these studies were primarily designed to detect differences in morphological end points and, therefore, utilised a time point of lesion stabilisation at which inflammatory and proliferative processes are poorly represented. More conclusive observations of the effect of ETB blockade on inflammatory aspects of lesion formation might be drawn from immunohistochemistry, flow cytometry or PCR for markers of macrophages (Mac2, F4/80) or other leucocytes (GR-1, CD45) at more representative, earlier time points – macrophage content peaks ~7 days after injury (MacDonald *et al*, Unpublished data) and neutrophil accumulation within hours (Roque *et al.*, 2000). Any effect on leucocyte adhesion might be determined by analysis of adhesion molecule (ICAM-1, VCAM-1, P-selectin) expression by the same techniques (Roque *et al.*, 2000; Sata *et al.*, 2000), or by imaging rhodamine-labeled leucocyte rolling and adhesion *in vivo*, by intra-vital microscopy (Osaka *et al.*, 2007). Similarly, any changes in αSMA expression or markers of cell proliferation such as BrdU incorporation might be more sensitive to detection at earlier time points – neointimal proliferation peaks at ~14 days in this model (Reis *et al.*, 2000). VSMC apoptosis, a possible candidate for ETB-mediated regulation of lesion size (Cattaruzza *et al.*, 2000; Lauth *et al.*, 2000), could also be determined at this time-point (neointimal apoptosis peaks at ~14 days) using immunohistochemistry (terminal deoxynucleotidyl transferase dUTP nick end labeling) or flow cytometry (annexin V assay). The origin of neointimal VSMC-like cells could be determined by transplantation of GFP-labelled bone marrow and subsequent GFP/αSMA co-localisation in injured vessels. Alteration of extracellular matrix content of neointimal lesions by ETB blockade might also regulate their size and could be identified by biochemical assays for collagen or elastin, or by determining the activity of matrix degrading enzymes by zymography.

Whilst such observational studies might provide evidence for a mechanism of action, firm conclusions are prevented by the complexity of interactions between cell types in the developing neointima. For example, an apparent effect of ETB blockade on VSMC proliferation might reflect alterations in the production of mitogenic cytokines by macrophages rather than a direct action on VSMC. The confirmation of any mechanism, therefore, requires specific intervention. The further use of Cre-*lox*P cell-specific recombination approach is an obvious solution to this problem. The cross of transgenic SM22-Cre mice with floxed ETB mice, would allow determination of the effect of ETB deletion specifically from VSMC on neointimal lesion formation to be determined. A similar approach to reveal the role of ETB in macrophages on neointimal lesion formation may also be adopted, by use of a Cre-recombinase construct driven by a macrophage specific promoter sequences such as that of M lysozyme (Clausen *et al.*, 1999). A potential caveat of this approach is the possibility that the progenitor-derived smooth muscle-like cells, which are prevalent in the neointima of wire-injury induced lesions (Sata *et al.*, 2002), may have abnormal expression of these markers during their lineage. Appropriate recombination would therefore need to be validated using Cre reporter animals. An alternative approach to determining the role of ETB in leucocytes on lesion formation would be to perform bone marrow transplantation studies between ETB deficient and ETB wild-type mice. In this case, however, deletion of ETB in bone marrow-derived neointimal VSMC-cells would be unavoidable (Sata *et al.*, 2002).

6.2.3 *Other processes of vascular remodelling*

Given the apparent protection against neointimal proliferation provided by ETB receptor activation, and the current debate over the superiority of ETA selective or non-selective ETA/B antagonists, further study of the effects of ETB blockade on other processes of vascular remodelling might be warranted. Atherosclerosis, for example, bears a number of similarities to neointimal hyperplasia, but results from chronic rather than acute injury to the vascular wall. ETA blockade has been shown to inhibit atherogenesis in apoE deficient mice (Barton *et al.*, 1998) and ETA/B blockade does so in LDL receptor deficient mice (Babaei *et al.*, 2000). Neither the effect of selective ETB blockade nor the comparison of ETA selective and ETA/B

blockade, however, has been reported. Whilst tolerability issues surrounding ET blockade (Battistini *et al.*, 2006) may mean ET antagonists are never likely to be administered solely for the prophylaxis of atherosclerosis, a beneficial anti-atherogenic effect may provide additional rationale for use of such agents in other conditions where they are already indicated. Thus, experiments similar to those performed here in which ET antagonists are administered to an animal model of atherogenesis (for example, high fat-fed apoE$^{-/-}$ mice) and resulting lesion size and stability investigated, might provide clinically relevant insights into the role of ETB in this process.

Angiogenesis is perhaps the ultimate form of vascular remodelling. It is considered important in the pathogenesis of several human conditions, including: cancer, myocardial infarction, macular degeneration and even atherosclerosis (reviewed by Carmeliet, 2003). ET-1 is thought to promote angiogenesis and induces an angiogenic phenotype in cultured endothelial cells by activation of ETB (Goligorsky *et al.*, 1999; Salani *et al.*, 2000; Kuhlmann *et al.*, 2005). In more complex systems, however, the role of this EC ETB pathway in angiogenesis is less clear (Pedram *et al.*, 1997; Matsuura *et al.*, 1998; Herrmann *et al.*, 2002), making this an ideal problem to address using cell-specific ETB deletion. Preliminary work in this area indicated that ET-1 was pro-angiogenic at low concentrations and inhibited the process at higher levels (**Figure 6-1**). Such experiments were complicated by a number of methodological issues, however, and as such, this strand of work was abandoned in favour of greater investment in vascular injury studies.

6.2.4 *Further development of OPT for vascular imaging*

Future development may also see the use of OPT for the examination other types of remodelling in small vascular structures. Atherosclerotic plaque formation, for example, is another widely-studied remodelling process that is also typically quantified by time-consuming histological analysis. Preliminary OPT scans of aortic arches from fat-fed apoE-/- mice clearly demonstrate atherosclerotic plaque with the predicted morphology and anatomical distribution (**Figure 6-2**). Therefore,

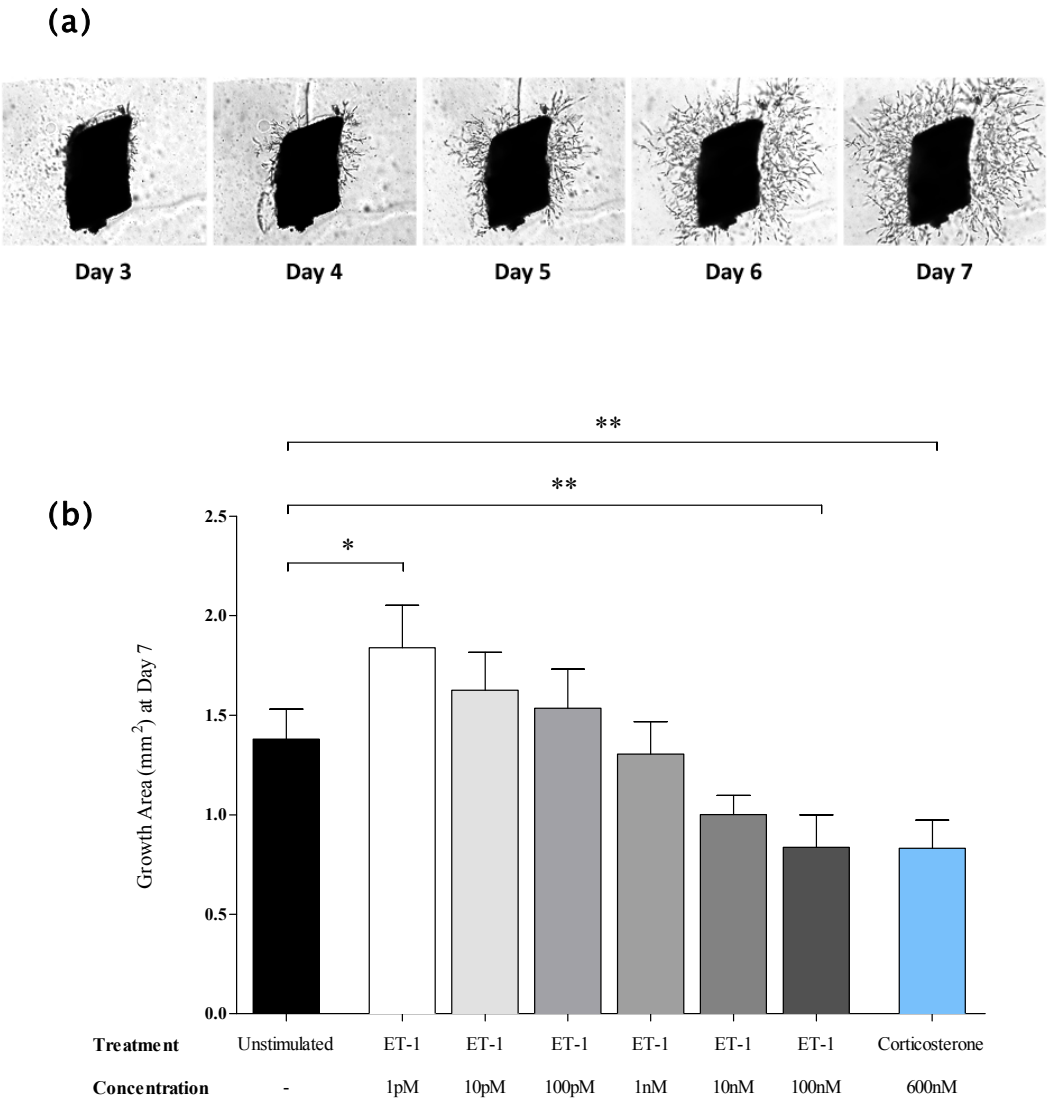


Figure 6-1 Impact of endothelin-1 treatment on angiogenesis from aortic ring explants.

Aortic rings were excised from male, 8-10 week old C57Bl6/J mice, cleaned of peri-adventitial material, embedded in matrigel basement membrane preparation and cultured in endothelial cell basal medium-2 containing vehicle, endothelin-1 (10^{-12}-10^{-7}M) or corticosterone ($6x10^{-7}$M). Medium and treatments were replaced every 2 days, and outgrowths allowed to develop for 7 days. The area of tube-like outgrowths from each ring was calculated from photomicrographs by morphometry. Progressive formation of tube-like structures from the periphery of aortic ring explants occurred from day 3, and the area of these peaked at day 7 (a). Treatment with endothelin-1 at the lowest dose (1pM) significantly augmented the area of outgrowth (b). At the highest tested dose, endothelin-1 (100nM) significantly reduced the area of outgrowth, to a similar extent as corticosterone (600nM). n = 6-12. Data are mean ± SEM. ns, *, $p>0.05$; **, $p<0.01$ by two-way ANOVA with Dunnet's post test.

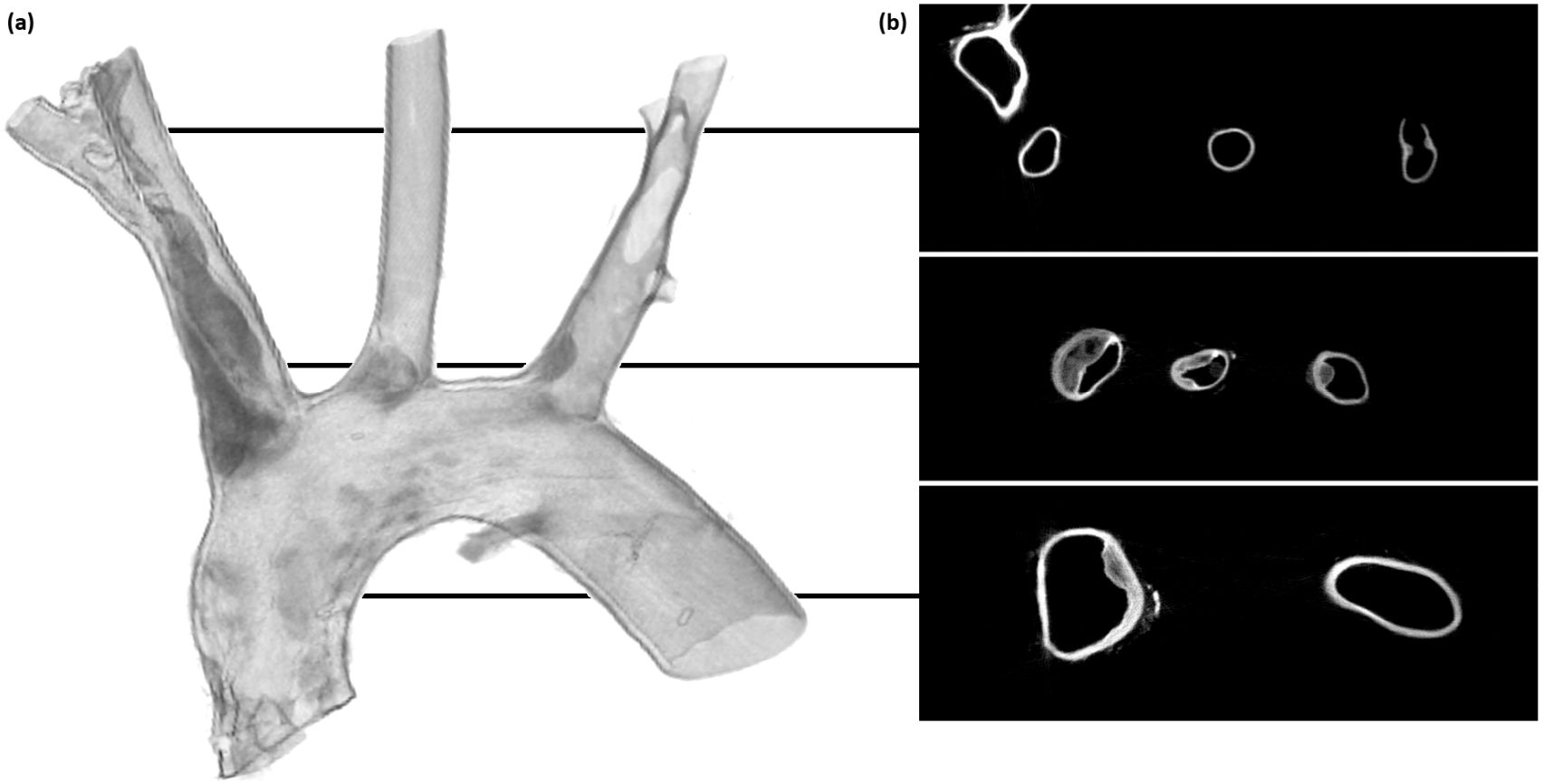


Figure 6-2 Optical projection tomographic imaging of atherosclerotic lesions in the mouse aortic arch.

Perfusion fixed, aortic arches from apolipoprotein E-deficient mice, fed on a western diet for 8 weeks, were subject to optical projection tomographic scanning by the methods developed for injured femoral arteries (section 2.5). In volume renderings of re-constructed tomograms, lesions were clearly visible in the brachiocephalic artery, the roots of the left common carotid and subclavian arteries and the lesser curvature of the aortic arch (a). In cross-section, these possessed atherosclerotic plaque-like morphology and could be distinguished from the media by their weaker fluorescent emission (b). Regions within these were devoid of fluorescence were commonly observed and may reflect sites of intra-plaque lipid pools, which would be predicted to be removed during sample preparation. Data provided by L. Low.

validation of OPT for quantification of these lesions may provide advantages of speed and 3D analysis to studies of this process. As the traditional refractive index matching solution used for OPT imaging is a lipid solvent, a polar substitute, such as glycerol, may be necessary to preserve plaque lipid content in this setting. Alternatively, as has been suggested for *ex vivo* MRI of atherosclerotic arteries, such removal of lipids may provide a means to estimate the volume of extracellular lipid pools within plaques by measurement of the empty regions of plaque that remain after treatment with solvent (McAteer *et al.*, 2004).

An additional potential development of the OPT technique is the use of whole-mount immunofluoresence to enable the imaging of volume and distribution of particular cell types or other antigens (Sharpe *et al.*, 2002; Alanentalo *et al.*, 2007). This was, again, the subject of preliminary investigations. These, however, yielded little success due to the difficulties of antibody penetration in whole tissues and non-specific binding of antibodies during the necessarily long reagent incubations. Further investigation of this possibility would require careful optimization of incubation and washing times, blocking reagent constitution and testing of alternative primary antibodies, for example, $F(ab)_2$ antibody fragments which may penetrate tissue more easily.

6.3 SUMMARY OF CONCLUSIONS

The role of ETA and ETB receptors in the remodelling that occurs following acute injury to the vascular wall was determined using selective pharmacological antagonists in two murine models of this process. These studies were extended to mice in which ETB receptors are selectively knocked-down in EC, thereby elucidating any role for ETB in EC without the confounding effects of ETB blockade in other cells. This work was intended to address the mechanism by which endogenous ETs modulate vascular remodelling and, therefore, to inform the clinical use of ETA selective and non-selective ETA/B antagonists in conditions in which similar processes may occur in man. The major findings of this project were:

- Complete ligation of the femoral artery is a robust model of neointimal hyperplasia and results in impairments to contractile function and endothelium-dependent vasodilatation, independent of neointima formation.
- OPT is a powerful technique for the rapid, non-destructive 3D evaluation of neointimal lesion size in small arteries
- ETA receptors mediate exacerbation of neointima formation following acute arterial injury
- ETB receptors moderate neointima formation following acute arterial injury through a mechanism other than blood pressure elevation, but this effect is dependent on the mode of vascular injury
- Concurrent ETB blockade prevents the beneficial effect of ETA blockade on neointima formation
- In the mouse femoral artery, ETB expressed by EC plays little role in modulating either vascular function, or neointima formation.

The results of these studies, therefore, indicate that the ETB receptor acts to moderate the detrimental effect of endogenous ETs on the ETA receptor in this setting of acute vascular injury. As such, selective ETA receptor antagonists may offer benefits over non-selective ETA/B antagonists in similar conditions in man. Further experiments are required to confirm and reveal how ETB receptors on non-EC cell types are able to moderate neointimal proliferation.

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Appendix

Publications

From: *Nicholas Kirkby*
Date: *14th July 2009*

Dear Sir/ Madam,

I am an author on a review article published in the British Journal of Pharmacology in March 2008:

"The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house?" Volume 153(6). Pages 1105-1119.

I would like to include a copy of this article in my PhD thesis, and would be grateful if you could supply me with an email confirming that this is acceptable.

Yours faithfully,

Nicholas Kirkby

From: Wade, Katie - Oxford **On Behalf Of** Permission Requests - UK
Date: 15th July 2009

Dear Nicholas Kirkby

Thank you for your email request. Permission is granted for you to use the material below for your thesis/dissertation subject to the usual acknowledgements and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis/dissertation commercially.

Kind Regards

Katie B Wade
Permissions Assistant
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